REMARKS

Claims 1-43 were pending in the instant application. Claims 1-14, 16-20, and 27-43 have been cancelled. New claims 44-47 have been added. Claims 15 and 21-26 are amended by the present Amendment. Support for the new claims and the amendments to the claims can be found in the claims as filed and in the original specification at least at page 2, lines 5-19 and page 39, lines 5-24. Upon entry of the present Amendment, claims 1-26 and 44-47 are pending and presented for reconsideration. Applicant respectfully submits that no new matter is introduced by the present Amendment.

Amendment and/or cancellation of the claims is not to be construed as acquiescence to any of the objections/rejections set forth in the instant Office Action or any previous Office Action of the parent application, and was done solely to expedite prosecution of the application. Applicant submits that claims were not added or amended during the prosecution of the instant application for reasons related to patentability. Applicant reserves the right to pursue the claims, as originally filed, or similar claims in this or one or more subsequent patent applications.

Claim Rejections - 35 U.S.C. §112

Rejection of Claims 1, 3-4, 6, and 8-14 under 35 U.S.C. §112, Second Paragraph

Claims 1, 3-4, 6, and 8-14 have been rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. In particular, the Office Action, on page 3, states that "Claim 1 [and claim 9] is [are] vague and indefinite in that it is unclear whether Applicant intends an anti-recombinase which is wild-type to a bacteriophage or whether Applicant intends an anti-recombinase which, *e.g.,* inhibits the bacteriophage recombinase recited in component (a) of the claim, or both." The Office Action also states that claims 3, 6, 10, and 12 lack antecedent basis for the limitation "the origin of replication" because "there can be more than one origin of replication in the claim from which it depends." Furthermore, the Office Action states that claims 4, 8, 11, and 14 are "vague and in define in that it is unclear what is meant by a bacteriophage λ anti-RecBCD." Finally, the Office Action states, on page 4, that there is "insufficient antecedent basis for this [nucleotide sequence] limitation in the claim because there is more than one sequence which encodes the bacteriophage λ Red recombinase in the claim from which claim 13 depends."

Without acquiescing to this rejection and solely in an effort to further prosecution, Applicant has cancelled claims 1-14, thereby rendering the rejection, as it pertains to these claims, moot. Applicant, therefore, respectfully requests withdrawal of the rejection of claims 1, 3-4, 6, and 8-14 under 35 U.S.C. §112, second paragraph and favorable reconsideration.

Rejection of Claims 1-26 under 35 U.S.C. §112, First Paragraph

Claims 1-26 have been rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. In particular, the Office Action, on page 7, states that, "no description is provided of a single Ptac promoter sequence. No description is provided of a recombinase other than λ Red. No description is provided of an anti-recombinase other than λ gam."

Without acquiescing to this rejection and solely in an effort to further prosecution, Applicant has cancelled claims 1-14 and 16-20, thereby rendering the rejection, as it pertains to these claims, moot. Not withstanding the foregoing, Applicant respectfully traverses the foregoing rejection. Reconsideration and withdrawal of the rejection in light of the following discussion is respectfully requested.

An objective standard for determining compliance with the written description requirement under 35 U.S.C. § 112, first paragraph, is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, the Applicant was in possession of the invention as now claimed. Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991) and In re Gosteli, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989).

Without acquiescing to this rejection and solely in an effort to further prosecution, Applicant has amended claim 15, and claims depending therefrom, to be directed to a recombinant organism comprising a PCR-mediated gene replacement vector that comprises: (a) a λ exo and a *λ bet* nucleotide sequence encoding bacteriophage λ Red recombinase; (b) a *λ gam* nucleotide sequence encoding bacteriophage anti-RecBCD; (c) a *Ptac* promoter sequence operably linked to the nucleotide sequence of (a) and (b); and (d) a nucleotide sequence encoding LacI operably linked to its native promoter; and (e) at least one origin of replication sequence which confers low copy number on the vector, wherein the recombinant organism is a

pathogenic species. Furthermore, the Examiner has admitted, at page 6 of the Office Action, that ***"[t]he specification describes nucleic acids for engineering bacterial chromosomes and teaches that the Red system, consisting of bet (a ssDNA annealing protein), exo (a 5'-3' dsDNA exonuclease) along with gam (an anti-RecBCD functioning protein) promote gene replacement into pathogenic bacteria***." In view of the foregoing, these amendments render the rejection, as it pertains to the recombinase and anti-recombinase sequences, moot.

With respect to the Ptac promoter sequence, Applicant respectfully submits that Applicant's specification also provides extensive teachings on the claimed Ptac nucleic acid sequences. To begin with, Applicant discloses that the Ptac promoter is present in the pKM200 and pKM201 vectors and was derived from pTP806 (see, *e.g.*, page 27, lines 25-32 of the specification). Furthermore, Applicant discloses in the specification on page 27, lines 18-22, that the plasmid, pTP806, which contains the Ptac promoter, was described previously by Poteete *et al.* (*Journal of Bacteriology*, 181(17):5402-5408, 1999), a reference that the Examiner has also cited (discussed in greater detail below). Additionally, the specification describes a Ptac-red-gam operon used for the expression of exo, bet, and gam sequences (see, *e.g.*, page 52, lines 17-18 of the specification).

In addition to the foregoing teachings in Applicant's specification, the art at the time of the invention is replete with teachings regarding the Ptac promoter. For example, as early as 1983, Amann *et al.*[1] described and cloned the Ptac promoter into a series of plasmid vectors to facilitate the expression of cloned genes (see, *e.g.*, Abstract). Pierucci *et al.*[2] had described the Ptac promoter for controlling gene expression (see, *e.g.*, Materials and Methods, first paragraph, and Abstract). Additionally, the Stewart reference (as cited in the Office Action at least at page 12) also teaches the use of a "commonly used strong promoter[s]", the Ptac promoter (see, *e.g.*, column 26, lines 13-16 of Stewart *et al.*).

Based on the foregoing teachings in Applicant's specification, as well as the general knowledge in the art at the time of the invention, one of skill in the art would understand the meaning of the term "Ptac promoter sequence," as used in the amended claims. Accordingly,

---

[1] A copy of which is attached herein as Appendix A.
[2] A copy of which is attached herein as Appendix B.

Applicant submits that amended claims 15 and 21-26 are clear and definite and respectfully request that the aforementioned rejection under 35 U.S.C. §112, first paragraph be reconsidered and withdrawn.

Claim Rejections - 35 U.S.C. §102

Rejection of Claims 1, 4, and 7-8 under 35 U.S.C. §102(b)

Claims 1, 4, and 7-8 have been rejected under 35 U.S.C. §102(b) being anticipated by Poteete *et al.* (*Journal of Bacteriology* 181(17):5402-5408, 1999). In particular, the Office Action, on page 10, states that, "Poteete *et al.* teach a vector comprising: (a) two nucleotide sequences encoding a bacteriophage recombinase (λ exo and bet); (b) a nucleotide sequence encoding a bacteriophage anti-recombinase (λ gam); a Ptac promoter sequence operably linked to the nucleotide sequences of (a) and (b): and a nucleotide sequence encoding LacI operably linked to its native promoter."

Without acquiescing to the validity of the Examiner's rejection and solely in the interest of expediting prosecution and allowance of the pending claims, Applicant has cancelled claims 1, 4, and 7-8. Thus, Applicant submits that this rejection has been rendered moot and respectfully request that the Examiner reconsider and withdraw this rejection.

Notwithstanding the foregoing, Applicants wish to make the following remarks of record.

For a prior art reference to anticipate a claimed invention, the prior art reference must teach each and every element of the claimed invention. Lewmar Marine v. Barient 827 F.2d 744, 3 USPQ2d 1766 (Fed. Cir. 1987).

The pending claims are directed to a recombinant organism comprising a PCR-mediated gene replacement vector that comprises: (a) a λ *exo* and a λ *bet* nucleotide sequence encoding bacteriophage λ Red recombinase; (b) a λ *gam* nucleotide sequence encoding bacteriophage anti-RecBCD; (c) a *Ptac* promoter sequence operably linked to the nucleotide sequence of (a) and (b); and (d) a nucleotide sequence encoding LacI operably linked to its native promoter; and (e) at least one origin of replication sequence which confers low copy number on the vector, wherein the recombinant organism is a pathogenic species, *e.g.*, enterohemorrhagic *E. coli* (EHEC) or enteropathogenic *E. coli* (EPEC), *Pseudomonas aeruginosa*, or *Micobacterium tuberculosis*. Thus, all of the pending claims require a recombinant organism comprising a

vector comprising a nucleotide sequence encoding LacI operably linked to its native promoter, wherein the recombinant organism is a pathogenic species.

As a first matter, Applicant wishes to point out that, contrary to the Examiner's assertions, the Poteete reference does *not* disclose a lacI sequence operably linked to its native promoter. For example, the description of pTP822 and pTP810 on page 5403, second column, second full paragraph of Poteete *et al.* describes vectors comprising the sequence P_{lac}-cI. The cI repressor gene of bacteriophage lambda, which is responsible for the regulation of the switch from lysogeny to lytic development in bacteriophage lambda, is completely distinct from the lacI gene. Furthermore, Poteete *et al.* further disclose that "[a]lthough the λ recombination and repressor genes in the substitution are nominally ***under the control of the wild-type lacI gene*** of this strain, effective expression is seen even in the absence of the inducer" (see, *e.g.*, page 5403, second column, second full paragraph of Poteete *et al.*). Thus, the Poteete reference discloses that the genes are under the control of the wild-type, *i.e.*, chromosomal, lacI gene, and that this wild-type chromosomal lacI gene is ***not a part of the vector***. Moreover, Applicant respectfully submits that this reference only discloses the use of laboratory strains of *E. coli* and is devoid of any teaching regarding a recombinant organism, *e.g.*, a ***pathogenic organism***, comprising vector sequences encoding a ***lacI gene operably linked to its native promoter.***

In summary, Applicant respectfully submits that, contrary to the Examiner's assertions, Poteete *et al.* fails to teach or suggest each and every element of the claimed invention and, thus, this reference fails to anticipate the claimed invention. For the foregoing reasons, rejection of the claimed invention is believed to be improper and Applicant respectfully requests that it be reconsidered and withdrawn.

Claim Rejections - 35 U.S.C. §103

Rejection of Claims 1-22 under 35 U.S.C. §103(a)

Claims 1-22 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Datsenko *et al.* (*PNAS* 97(12):6640-6645, 2000) in view of Stewart *et al.* (U.S. Patent No. 6,355,412). In particular, the Office Action states on page 13 that, "[i]t would have been obvious for one of ordinary skill in the art to combine the P_{tac} promoter of Stewart *et al.* in the

vector of Datsenko *et al.* because both Stewart *et al.* and Datsenko *et al.* teach methods of recombinatorial engineering in bacteria which rely upon the expression of λ exo and bet and gam sequences." The Office Action further states that "[o]ne of ordinary skill in the art would have been motivated to combine the vector comprising the λ exo and bet and gam sequences as taught by Datsenko *et al.* with the P_{tac} promoter and lacI gene sequences as taught by Stewart *et al.*"

Applicant respectfully traverses the Examiner's assertion that the claimed invention would have been obvious to the skilled artisan at the time it was made. Reconsideration and withdrawal of the rejection in light of the following discussion is respectfully requested.

To establish a prima facie case of obviousness for the claimed invention, there must have been some suggestion or motivation, either in the cited references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings in the manner proposed by the Examiner. Second, there must have been a reasonable expectation of success at the time the invention was made. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. See M.P.E.P. 2143. The prior art must suggest "to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process" and "[b]oth the suggestion and the reasonable expectation of success must be founded in the prior art, not in the applicant's disclosure." In re Dow Chemical Co. 837 F.2d 469. 473, 5 U.S.P.Q.2d 1529, 1531 (Fed.Cir. 1988).

Applicant submits that the Examiner has failed to establish a *prima facie* case of obviousness since the cited references would not have motivated one of ordinary skill in the art to arrive at the claimed invention.

The amended claims are directed to a recombinant organism comprising a PCR-mediated gene replacement vector that comprises: (a) a λ exo and a *λ bet* nucleotide sequence encoding bacteriophage λ Red recombinase; (b) a *λ gam* nucleotide sequence encoding bacteriophage anti-RecBCD; (c) a *Ptac* promoter sequence operably linked to the nucleotide sequence of (a) and (b); and (d) a nucleotide sequence encoding LacI operably linked to its native promoter; and (e) at least one origin of replication sequence which confers low copy number on the vector, wherein the recombinant organism is a ***pathogenic species, e.g.***, enterohemorrhagic *E. coli* (EHEC) or enteropathogenic *E. coli* (EPEC), *Pseudomonas aeruginosa*, or *Micobacterium tuberculosis*. In contrast, Datsenko *et al.* neither teach nor suggest all of the elements of the claimed invention, alone or in combination with the teachings of Stewart *et al.*

Datsenko *et al.* is directed to the PCR-mediated gene replacement of chromosomal genes in *E. coli* K-12, a non-pathogenic strain of *E. coli*. Datsenko *et al.* teach the use of an isolated nucleic acid comprising λ *bet, exo* and *gam* sequences under the control of an arabinose-inducible promoter (P_{bad}) in a low copy number vector (*i.e.,* isolated nucleic acid molecule), further comprising a temperature sensitive origin of replication, allowing for easy elimination of the vector at 37°C, with an optimized ribosome-binding site for efficient translation of *gam*. This reference is devoid of any teaching regarding a recombinant organism, *e.g.,* a pathogenic organism, comprising sequences encoding λ Red Recombinase sequences under the control of a P*tac* promoter or a *lacI* gene operably linked to its native promoter.

The teachings of Stewart *et al.* fail to cure this deficiency. Stewart *et al.* is directed to methods and compositions for directed cloning and subcloning using homologous recombination (see, *e.g.,* Title and Abstract of Stewart *et al.*). The Stewart reference discloses an isolated nucleic acid comprising the recombinase λ Red α (*exo*) and Red β (*bet*) genes and that levels of expression can also be varied by using promoters of different strengths, including but not limited to, the tac promoter. However, the Stewart reference is devoid of any teaching regarding the use of homologous recombination for PCR-mediated gene replacement, *i.e.,* to create genetic knock-outs, in pathogenic strains, as required by the pending claims. Thus, even when combined, the teachings of the Datsenko *et al.* and Stewart *et al.* would fail to even suggest to one of ordinary skill a recombinant organism comprising a PCR-mediated gene replacement vector, wherein the recombinant organism is a pathogenic species, as presently claimed.

Moreover, Applicant respectfully submits that the ordinary artisan would not have been motivated to further combine the teachings of Datsenko *et al.* and Stewart *et al.* to create the recombinant organism, as presently claimed. Specifically, although both references utilize the λ Red recombinase system, Datsenko *et al.* is directed to the one-step inactivation of chromosomal genes in a non-pathogenic strain of *E. coli*, while Stewart *et al.* is directed to cloning and subcloning using homologous recombination. The Office Action has failed to point to any teaching in the cited references which would impel one of ordinary skill in the art to combine the teachings of the references in order to arrive at the presently claimed invention. It is well-established law that the prior art must suggest "to those of ordinary skill in the art that they

should make the claimed composition or device, or carry out the claimed process" and "[b]oth the suggestion and the reasonable expectation of success ***must be founded in the prior art, not in the applicant's disclosure*** (emphasis added)." *In re Dow Chemical Co.* 837 F.2d 469. 473, 5 U.S.P.Q.2d 1529, 1531 (Fed.Cir. 1988). Thus, absent evidence to the contrary, the combination of the cited references amounts to an attempt at hindsight reconstruction of the claimed invention based on the teachings of Applicant's own specification and is clearly impermissible. See, for example, *In re Fine* 5 USPQ2d 1596 (Fed.Cir. 1988); *In re Gorman* 18 USPQ2d 1885 (Fed. Cir. 1991); *In re Fitch* 23 USPQ2d 1780 (Fed. Cir. 1990).

Applicant further submits that, even if the motivation to combine the teachings of the references did exist, which Applicant denies, Datsenko *et al.* in combination with Stewart *et al.* fail to provide sufficient information that would lead one of ordinary skill to reasonably expect that these vectors could be successfully used to inactivate chromosomal genes in ***pathogenic bacteria***. The present invention features improved methods and systems for promoting recombination in bacteria, specifically in pathogenic strains of bacteria. The Office Action states, on page 11, that "Datsenko *et al.* explicitly teach such a nucleic acid in an *E. coli* K12 recombinant host, but note that their method should be 'widely useful' and easily extended to use in other bacteria." Contrary to this statement, there was no reasonable expectation of success in achieving recombination in ***pathogenic strains*** at the time of the claimed invention. At the time of the instant invention, ***the known λ Red systems, i.e., the λ Red taught by Datsenko et al. or Stewart et al. could not be used successfully in pathogenic bacterial strains***, as required by the pending claims. For example, as described in the instant application on page 39, lines 17-20, "***initial attempts by the instant inventors to employ λ Red,"*** **(*i.e.*, the λ Red taught by Datsenko *et al.* and/or Stewart *et al.*),** ***"for PCR-mediated gene replacement at various loci in [the pathogens] EHEC and EPEC were met with sporadic success***, similar to the limited success seen with Red-promoted short homology recombination in *Y. pseudotuberculosis*." It was these difficulties that prompted the inventors to examine more closely the methodologies of λ Red promoted PCR-mediated gene replacement, especially in regard to optimizing its use in the pathogenic organisms EHEC and EPEC. The specification further states on page 53, lines 26-28, that, in contrast to known λ Red systems, "[t]he above Examples demonstrated that λ Red can be utilized for the manipulation of the chromosomes of [the pathogens] EHEC and EPEC" and that "the ability to inactivate or replace a gene of interest

in the chromosomes of bacterial pathogens is a critical step in the identification of virulence factors." To summarize, at the time of the instant invention, the use of known λ Red systems, including the λ Red taught by Datsenko *et al.* or Stewart *et al.* for PCR-mediated gene replacement in pathogenic species led only to inefficient and sporadic results. ***The limited success of the previous λ Red systems (i.e., the λ Red taught by Datsenko et al. and/or Stewart et al.), led the inventors to select the specific combination of recombinase, anti-recombinase, Ptac promoter, and lacI presently claimed to improve efficiency of Red recombineering in pathogenic strains.***

In summary, Applicant respectfully submits that, contrary to the Examiner's assertions, the ordinarily skilled artisan at the time of Applicant's invention would not have been motivated nor have reasonably expected to succeed in arriving at Applicant's invention based on the teachings of Datsenko *et al.* and/or Stewart *et al.* The claimed invention pertains to isolated nucleic acid molecules comprising the recombination genes of phage λ *exo*, *bet* and *gam* operably linked to a P*tac* promoter sequence, and the use of a *lacI* gene in order to induce expression of the recombinase. These molecules are not taught or suggested in the cited art. Therefore, Applicant respectfully requests withdrawal of the rejection of claims 1-22 under 35 U.S.C. §103(a) and favorable reconsideration.

Rejection of Claims 1-26 under 35 U.S.C. §103(a)

Claims 1-26 have been rejected under 35 U.S.C. §103(a) as being unpatentable over Datsenko *et al.* in view of Stewart *et al.* and further in view of Pelletier *et al.* In particular, the Office Action states on page 14 that, "Pelletier *et al.* teach a method for identifying suitable targets for antibacterial agents based on identifying targets of bacteriophage-encoded proteins... that *Pseudomonas aeruginosa* is an exemplary pathogen which can be infected by bacteriophage... [and] *Mycobacterium tuberculosis* is an exemplary pathogen." Furthermore, the Office Action states on page 15 that, "[i]t would have been obvious for one of ordinary skill in the art to place the vector as taught by Datsenko *et al.* in view of Stewart *et al.* into hosts such as *P. aeruginosa* and *M. tuberculosis* as taught by Pelletier *et al.* because Datsenko *et al.* and Stewart *et al.* teach that the vector can be used in other bacteria, and Pelletier *et al.* teach that *P. aeruginosa* and *M. tuberculosis* are pathogenic bacteria subject to infection/manipulation by bacteriophage sequences."

Applicant respectfully traverses the Examiner's assertion that the claimed invention would have been obvious to the skilled artisan at the time it was made. Reconsideration and withdrawal of the rejection in light of the following discussion is respectfully requested.

The legal requirements to establish a *prima facie* case of obviousness are set forth above. Applicant submits that the Examiner has failed to establish a *prima facie* case of obviousness since at the time the invention was made there was no motivation to combine the references in the manner suggested by the Examiner, nor was there a reasonable expectation of success in making the claimed invention. The teachings of Datsenko *et al.* and Stewart *et al.* are set forth above. As discussed previously, the Examiner has not provided the requisite motivation to combine the Stewart and Datsenko references. In addition, based on the teachings of the references, there was no reasonable expectation of success in making the claimed invention based on the teachings of these references.

Pelletier *et al.* disclose a method for identifying suitable targets for antibacterial agents based on identifying targets of bacteriophage-encoded proteins by contacting a bacterial protein with a bacterial growth inhibitory bacteriophage polypeptide, determining whether said protein binds to said polypeptide, and identifying any bound protein (see, *e.g.*, Abstract and Claim 1 of Pelletier *et al.*). Pelletier *et al.* do not disclose the use of homologous recombination or gene replacement vectors. Furthermore, Pelletier *et al.* do not disclose the λ exo and a *λ bet* nucleotide sequence that encodes bacteriophage λ Red recombinase or the *λ gam* nucleotide sequence encoding bacteriophage anti-RecBCD. Thus, Pelletier *et al.* fails to provide the motivation required to modify the teachings of the cited art to arrive at the claimed invention.

Absent the teachings of the instant application, there was no motivation to make a pathogenic oragnism comprising specific constructs, as claimed. The specific constructs recited in the claims consistently allow for efficient recombination to inactivate chromosomal genes in ***pathogenic organisms***. More specifically, there was no motivation to combine a P_{tac} promoter, recombinase sequences, an anti-recombinase sequence, and an origin of replication which confers low copy number and temperature sensitivity with the pathogenic bacteria of Pelletier *et al.* absent the teachings of the instant application.

In view of the foregoing, Applicant respectfully submits that, contrary to the Examiner's assertions, the ordinarily skilled artisan at the time of Applicant's invention would not have been motivated nor have reasonably expected to succeed in arriving at Applicant's invention based on

the teachings of Datsenko *et al.*, Stewart *et al.*, and/or Pelletier *et al.* Therefore, the claimed invention is not obvious in view of the cited art. Applicant respectfully requests withdrawal of the rejection of claims 1-26 under 35 U.S.C. §103(a) and favorable reconsideration.

CONCLUSION

In view of the foregoing, entry of the amendments and remarks presented, favorable reconsideration and withdrawal of the rejections, and allowance of this application with the pending claim are respectfully requested. If a telephone conversation with the Applicant's attorney would expedite prosecution of the above-identified application, the Examiner is invited to call the undersigned at (617) 227-7400.

Dated: January 8, 2007

Respectfully submitted,

By _____

Megan E. Williams
Registration No.: 43,270
LAHIVE & COCKFIELD, LLP
One Post Office Square
Boston, Massachusetts 02109-2127
(617) 227-7400
(617) 742-4214 (Fax)
Attorney/Agent For Applicant

Gene, 25 (1983) 167–178
Elsevier

GENE 864

Vectors bearing a hybrid *trp-lac* promoter useful for regulated expression of cloned genes in *Escherichia coli*

(Recombinant DNA; pBR322; *c*I repressor overproduction; eukaryotic protein overproduction)

Egon Amann *, Jürgen Brosius * and Mark Ptashne

Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138 (U.S.A.) Tel. (617) 495-2352

(Received December 23rd, 1982)
(Revision received April 12th, 1983)
(Accepted May 5th, 1983)

SUMMARY

A strong promoter has been cloned on a series of plasmid vectors that facilitate the expression of cloned genes. This promoter, named *tac* [first described by DeBoer et al. (in Rodriguez, R.L. and Chamberlin, M.J. (Eds.),Promoters, Structure and Function. Praeger, New York, 1982, pp. 462–481)] contains the –10 region of the *lac*UV5 promoter and the –35 region of the *trp* promoter. Our vectors contain various cloning sites followed by transcription termination signals. In addition, we describe plasmids that facilitate the conversion of the *lac* promoter to the stronger *tac* promoter. Thus, preexisting gene fusions using the *lac* or the *lac*UV5 promoter can be readily converted to *tac* promoter gene fusions without changing the ribosome-binding site (RBS). The *tac* promoter is repressed in *lacI*^{Q} strains and can be induced by isopropylthio-β-D-galactoside (IPTG). Studies of expression of the *c*I repressor of bacteriophage λ show that the *tac* promoter is at least five times more efficient than the *lac*UV5 promoter. Under optimal conditions λ repressor constitutes up to 30% of the total cellular protein.

INTRODUCTION

Promoters are segments of DNA which direct RNA polymerase binding and initiation of transcription. At present, it is difficult to correlate the strength of a promoter with its primary structure even though more than 50 promoter sequences have been determined (Siebenlist et al., 1980; Rosenberg and Court, 1979). Comparison of these sequences, however, has revealed two regions of homology, the Pribnow box or –10 region (Pribnow, 1975; Schaller et al., 1975) with consensus sequence TATAAT, and the –35 region (Maniatis et al., 1975; Gilbert, 1976), with consensus sequence TTGACA. Promoter mutations map almost exclusively in either of these two domains (Siebenlist et al., 1980; Rosenberg and Court, 1979; Youderian et al., 1982).

The *lac*UV5 promoter contains the consensus –10 sequence and the *trp* promoter contains the

* Present addresses: (E.A.) Behringwerke Aktiengesellschaft, Postfach 1140, D-3550 Marburg 1 (F.R.G.) Tel. 49-6421-392961; (J.B.) Center for Neurobiology and Behavior, Columbia University, 722 West 168th Street, New York, NY, 10032 (U.S.A.) Tel. (212) 9602245 or 9602259.

Abbreviations: amp, ampicillin; bp, base pairs; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria broth; RBS, ribosome-binding site (Shine-Dalgarno sequence); SDS, sodium dodecyl sulfate.

consensus −35 sequence (Siebenlist et al., 1980; Rosenberg and Court, 1979). These elements can be fused using restriction sites *Taq*I and *Hpa*II to yield a hybrid ("*tac*") promoter as first shown by DeBoer et al. (1982; 1983). Recent reports indicate the importance of the spacing between the −10 and −35 promoter regions (Mandecki and Reznikoff, 1982; Stefano and Gralla, 1982, Russell and Bennett, 1982). In all the known, sequenced, promoters spacings of 15 to 18 bp are found, the most frequent interval being 17 bp (Siebenlist et al., 1980; Rosenberg and Court, 1979). The spacing in the *lac* and *trp* promoters is 18 and 17 bp, respectively. The *tac* promoter construction spaces the two domains 16 nucleotides apart, the same as that found in the strong ribosomal RNA promoters (Brosius et al., 1981).

The *tac* promoter includes the *lac* operator region and can therefore be repressed by *E. coli* strains that overproduce the *lac* repressor. Repressibility is important to allow the controlled production of proteins that are toxic to *E. coli*. The promoter can be induced to its full strength by addition of IPTG to the medium.

Here we report *tac* promoter constructions in expression vectors, which bear unique cloning sites downstream of the promoter and of DNA sequences encoding the *lacZ* RBS. Furthermore, as strong promoters tend to destabilize plasmids (Gentz et al., 1981), we have placed distal to the cloning sites several efficient signals for the termination of transcription. We have studied expression of a prokaryotic gene and show that the *tac* promoter is significantly more efficient than the *lac*UV5 promoter.

MATERIALS AND METHODS

(a) Bacterial strains and plasmids

The standard strain for carrying *tac* promoter containing plasmids was *E. coli* RB791 (= W3110 *lacI*^{Q}L8) (Brent and Ptashne, 1981). For some experiments strains MM294 (*pro*$^{-}$, *thi*$^{-}$, *endA*$^{-}$, *hsr*$_{k}^{-}$, *hsr*$_{m}^{+}$) (Lauer et al., 1981), X90[F′*lacI*^{Q1}] (kindly provided by H. Shuman) and LG90 (F^{-}, *Δlac proX*III) (Guarente et al., 1980) were used as indicated in the text. Plasmids pKB277, pKB280 and pGL101 are described elsewhere (Lauer et al., 1981; Backman and Ptashne, 1978). Plasmid p*lacI*^{Q}, which contains a 1.1-kb *lacI* fragment in the *Eco*RI site of pBR322, was kindly provided by J. Wang.

(b) Manipulation of DNA

Isolation of plasmid DNA, preparation of DNA fragments, DNA synthesis reactions, DNA ligations and transformation of *E. coli* were carried out as described previously (Backman et al., 1976; Talmadge and Gilbert, 1980; Maxam and Gilbert, 1980; Mandel and Higa, 1970). Plasmid-containing colonies were screened by the method of Birnboim and Doly (1979). DNA sequencing was performed according to Maxam and Gilbert (1980). Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, Inc. and from Boehringer, Mannheim and were used according to the manufacturers instructions. *Hin*dIII linkers (decamer: 5′CCAAGCTTGG) were purchased from Collaborative Research, Inc.

(c) Isolation of the 192-bp *trp* −35 fragment

Plasmid ptrpH1 (Fig. 1) was digested with *Pst*I and *Hin*dIII and the 1650-bp fragment was isolated and cut with *Taq*I. Four subfragments of approx. 1000, 400, 200 and 40 bp were identified by gel electrophoresis. Upon "shotgun-cloning" these fragments into the *Cla*I site of pBR322, only the 400- and 200-bp fragments were recovered, indicating that these were internal *Taq*I fragments. A *Hin*cII site at position −37 to −32 in the *trp* promoter (Bennett et al., 1978) identified the 200-bp fragment as the one carrying the −35 *trp* promoter sequence. DNA sequencing showed this fragment to be 192 bp in length (Fig. 4).

(d) Isolation of the 55-bp *lac*UV5 −10 fragment

Plasmid pGL101 (Lauer et al., 1981) was digested with *Hin*dIII and *Pvu*II. The 550-bp fragment was isolated, digested with *Hpa*II and the 55-bp fragment was isolated.

(e) Selection of λ-immune colonies

Fusion constructions of *tac* promoter-cI (see RESULTS, section a) were done as follows: Gel-puri-

fied DNA fragments were ligated, transformed into strain W3110*lacI*^{Q}L8 and plated on LB-plates containing 50 μg/ml ampicillin seeded with 10^9 λKH54 phage (Blattner et al., 1974) and 10^9 λh80KH54 phage. Plasmid-conferred immunity was further tested by streaking single colonies across a streak of phage on an agar plate. λ-immune, λ*imm*434-sensitive colonies were used for plasmid preparations and induction curves (see legends to Figs. 6 and 7).

(f) Electrophoretic analysis of proteins

1-ml samples of cultures grown in LB medium were harvested by centrifugation and the cell pellets were lysed in 100 μl of sample buffer according to Laemmli (1970). Samples were boiled for 5 min prior to loading, and 10–20 μl per well were analyzed by electrophoresis on 13.5% SDS-polyacrylamide gels. Photographs of Coomassie blue-stained gels were scanned with a Quick Scan R&D microdensitometer of Helena Laboratories.

RESULTS

(a) Construction of plasmids containing a hybrid *trp-lac* promoter

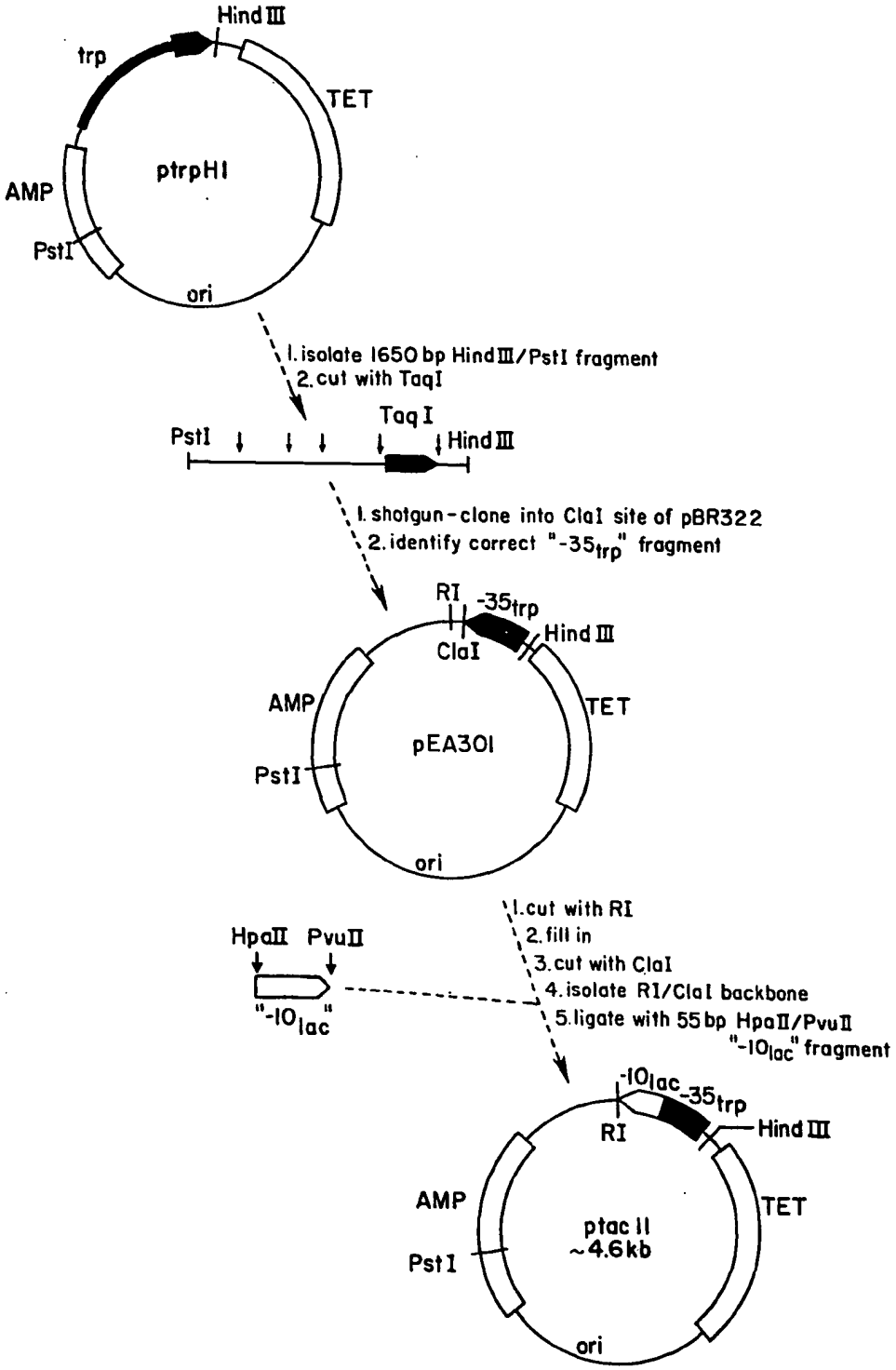
Plasmids ptac11 and ptac12 contain the *tac* promoter (with the *lac* operator and the *lacZ* RBS sequence) oriented in opposite directions with regard to the *amp* gene, followed by unique cloning sites (Figs. 1 and 2). In ptac11 a unique *Eco*RI site is located 7 bp downstream of the encoded *lacZ* RBS sequence and in ptac12 a unique *Pvu*II site is located 5 bp downstream of the encoded *lacZ* RBS sequence (Figs. 1 and 2). Following linearization of ptac12 with *Pvu*II and religation in the presence of *Hin*dIII linkers, ptac12H was constructed which carries a unique *Hin*dIII site 10 bp downstream from the *lacZ* RBS sequence. These sites are useful for the introduction of cloned genes and the formation of "hybrid ribosome binding sites" (Backman and Ptashne, 1978; see also DISCUSSION).

(b) Construction of plasmids that allow the conversion of *lac* promoter into *tac* promoter

The construction of the hybrid *tac* promoter was facilitated by the presence of a *Taq*I site located between the −10 and −35 *trp* promoter region and a *Hpa*II site in the corresponding position in the *lac*UV5 promoter. These sites have complementary ends and therefore can be fused. Before fusing *Taq*I and *Hpa*II sites, however, we converted the *Taq*I site at the end of a fragment bearing the −35 *trp* promoter region to a *Cla*I site (Fig. 3). The *Cla*I site when cut bears the same sticky ends as the *Taq*I and *Hpa*II sites but is unique to the plasmid. Thus, in plasmids pEA300 and pEA301, the immediate progenitors of ptac11 and ptac12 (see Figs. 1 and 2), cleavage with *Cla*I produces a fragment that bears the −35 *trp* promoter region at one end. This is useful for converting *lac* promoters into *tac* promoters. Cleavage with *Hpa*II of a DNA molecule carrying a *lac* promoter followed by a gene and insertion of the appropriate fragment into the *Cla*I site of pEA300 or pEA301 in the correct orientation yields the *tac* promoter without changing the RBS.

In the construction of pEA301, as described in Fig. 1, all of the clones isolated bore the −35 *trp* promoter fragment in the orientation shown in the Fig. 1. Had this fragment been recovered in the opposite orientation it would have formed a hybrid *trp-tet* promoter with a spacing between the −35 *trp*

Fig. 1. Construction of plasmids pEA301 and ptac11. Plasmid ptrpH1 carries the *trp* promoter but is deleted for the *trp* attenuator region (E. Amann and M. Ptashne, unpublished). The *trp* −35 promoter region was isolated on a 192-bp *Taq*I fragment from ptrpH1 by shotgun-cloning into the *Cla*I site of pBR322 (see MATERIALS AND METHODS, section c). Ligation with the *Taq*I (5'TCGA) fragment recreated one *Cla*I site (5'ATCGAT), located between the −35 and −10 *trp* promoter regions and its adjacent 5' base (Bennett et al., 1978; see also Figs. 3 and 4). We only detected clones in which the −35 *trp* promoter region was oriented in the same direction as the *amp* promoter of pBR322. Thus in pEA301 the *Cla*I site is located 23 bp from the *Eco*RI site. As a source of the *lac*UV5 −10 promoter region we used a 55-bp *Hpa*II-*Pvu*II fragment from plasmid pGL101 (Lauer et al., 1981). This fragment also carries the *lacZ* RBS sequence. pEA301 was cut with *Eco*RI and filled in and cut with *Cla*I. Ligation of the large fragment with the 55-bp *Pvu*II-*Hpa*II fragment yielded ptac11. AMP (ampicillin) and TET (tetracycline) mark the antibiotic resistance genes (open boxes) on the plasmids.



The thin lines mark the remaining pBR322 sequences. The line of medium thickness corresponds to sequences near the *trp* operon. The solid arrow refers to the *trp* promoter or part there of including its orientation. The open arrow marks the −10 part of the *lac*UV5 promoter.

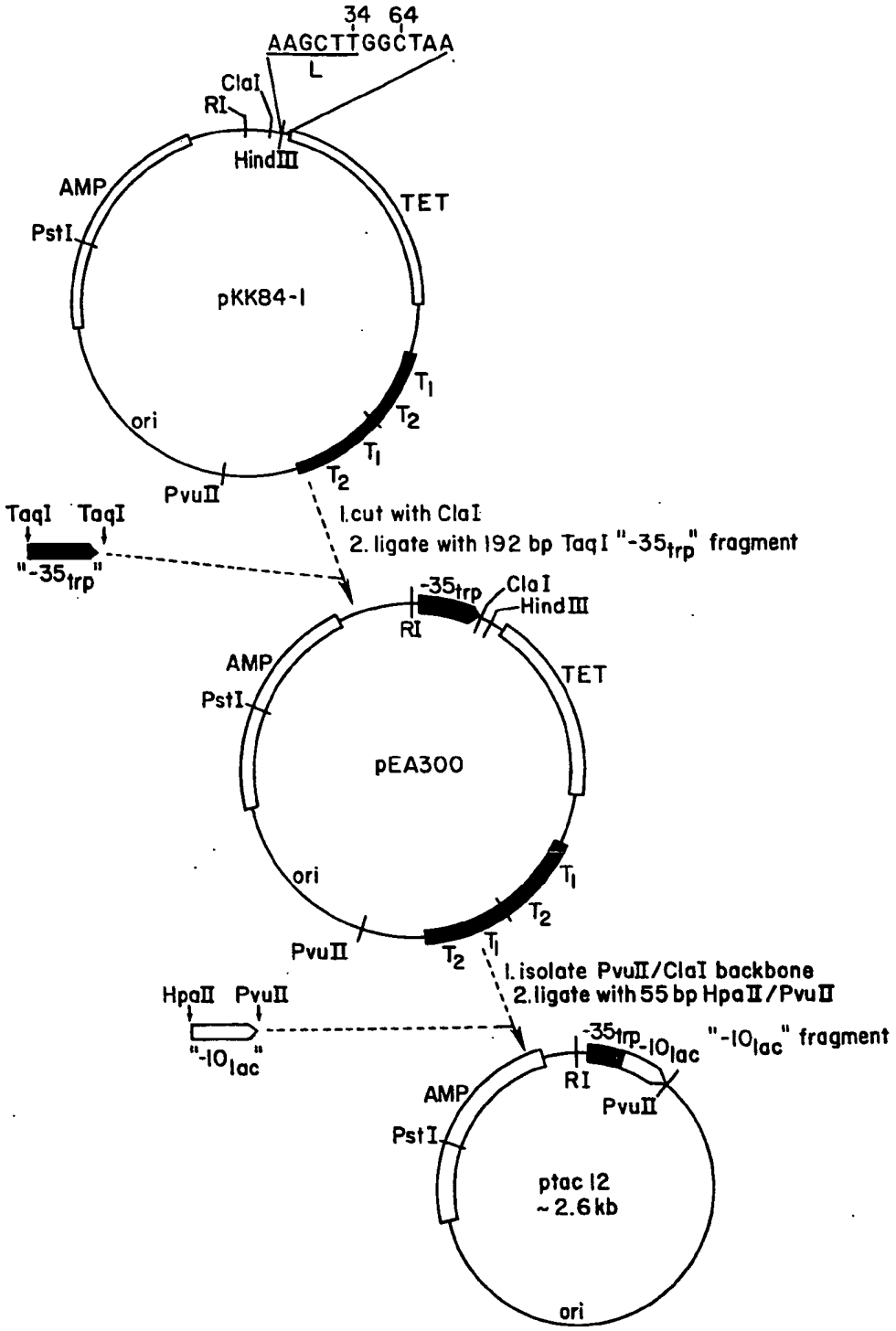


Fig. 2. Construction of plasmids pEA300 and ptac12. Insertion of the 192-bp *trp* promoter *Taq*I fragment (black arrow) into the *Cla*I site of plasmid pKK84-1 (J. Brosius, unpublished) yielded pEA300. Plasmid pKK84-1 carries a deletion of residues 34–64 of the standard pBR322 map (Sutcliffe, 1979) and contains two tandem inserts of a 500-bp fragment inserted into the filled-in *Ava*I site of pBR322. Each 500-bp fragment (black heavy segment) contains the two transcription terminators (T_1 and T_2) of the *rrnB* operon and the 5S gene (Brosius et al., 1981). Ligation of the 55-bp *Hpa*II-*Pvu*II −10 *lac*UV5 promoter fragment (see legend to Fig. 1) with the *Cla*I-*Pvu*II backbone of pEA300 yielded ptac12. AMP (ampicillin) and TET (tetracycline) mark the antibiotic resistance genes (open boxes) on the plasmids. The thin lines mark the remaining pBR322 sequences. The open box with the arrow corresponds to the *lac*UV5 −10 promoter region which is fused to the *trp* −35 region (black segment in ptac 12). *Eco*RI restriction sites are abbreviated as RI. L denotes the *Hin*dIII recognition sequence on the *Hin*dIII linker CCAAGCTTGG which has been fused to position 64 of pBR322 (Sutcliffe, 1979).

promoter region and the −10 *tet* promoter region of 17 bp. We failed to find such plasmids in any of three experiments: (*i*) selection of ampicillin-resistant clones; (*ii*) selection on low tetracycline (5 μg/ml) plates, and (*iii*) selection on high tetracycline (50 μg/ml) plates. We do not understand why in 36 isolates (on ampicillin selection) the *Taq*I fragment was in one orientation only.

We were able to obtain the −35 *trp* promoter fragment in the opposite orientation in pEA300 by cloning the fragment into plasmid pKK84-1 (Fig. 2). This plasmid carries a deletion in the *tet* promoter region but maintains the *Cla*I site (see legend to Fig. 2). The fragment was found to be inserted in both orientations with equal frequency.

(c) Plasmids which overproduce the *c*I repressor protein under *tac* promoter control

A series of plasmids have been constructed in this laboratory which overproduce the phage λ *c*I repressor protein under the control of the *lac*UV5 promoter (Backman and Ptashne, 1978; Backman et al., 1976). The most efficient overproducers of this series (pKB277, pKB280) contain a "hybrid ribosome-binding site", i.e. the RBS sequence of *lacZ* is fused to the ATG of the *c*I gene.

To compare the strength of the *lac*UV5 and *tac* promoters, we exchanged the −35 *lac*UV5 promoter region of pKB277 with the corresponding −35 *trp* promoter region, thereby generating the *tac* promoter as follows: pKB277 was digested with *Hpa*II and the 1100-bp fragment containing the *lac*UV5 −10 sequence, the hybrid ribosome binding site and the entire *c*I gene was ligated to *Cla*I-digested plasmids pEA300 and pEA301 (See Figs. 1 and 2). Cells were transformed and λ-immune colonies were selected. All colonies thus obtained contained inserts only in that orientation which generated the *tac* promoter upstream from the *c*I gene. In plasmid pEA302 the *tac* promoter transcribes in the counterclockwise direction and in pEA305 in the clockwise direction. Both these plasmids contain a single copy of the 1100-bp *Hpa*II fragment. One plasmid, pEA303, was recovered that contains two *Hpa*II fragment inserts as direct repeats in the clockwise orientation with respect to the *tac* promoter. Thus, this isolate constitutes one *tac* promoter transcribing two *c*I genes, each of which is preceded by a *lac* operator. Plasmids pEA302, pEA303, and pEA305 were used for expression experiments (see below and Fig. 6).

Plasmids pEA302 and pEA305 have the same hybrid ribosome binding site as pKB277 and pKB280 (Backman and Ptashne, 1978) but upstream from this region they have the *tac* promoter instead of the *lac*UV5 promoter. However, the plasmid structures of pEA302 and pEA305 differ from each other as well as from pKB277 and pKB280. To

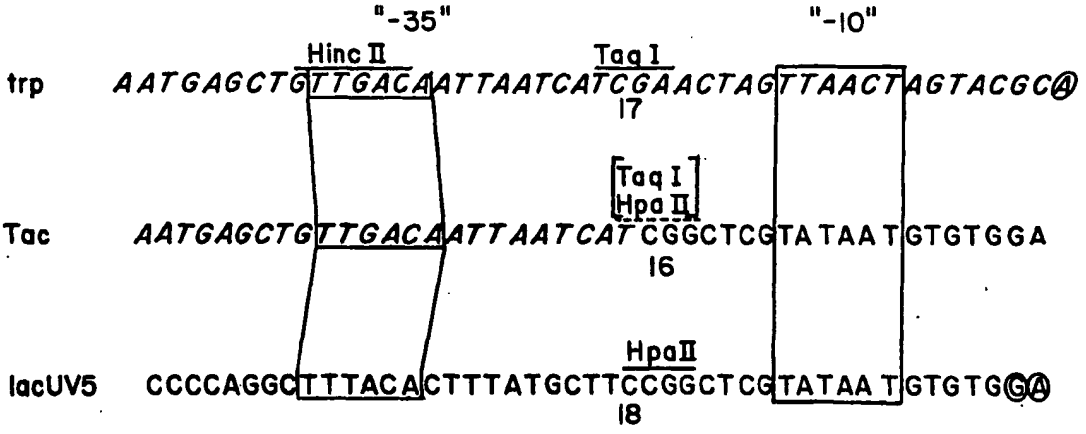


Fig. 3. Comparison of *trp*, *lac*UV5 and *tac* promoter sequences. For sequence determination of the *tac* promoter, plasmids ptac11 (Fig. 1) and ptac12 (Fig. 2) were linearized with *Eco*RI and labeled with ^{32}P by the "fill-in-reaction" according to Maxam and Gilbert (1980). After a secondary cut with *Hin*dIII or *Pvu*II, respectively, the promoter fragment was isolated and thus the sequence determined in both orientations. Sites used for the fusions are indicated (*Taq*I in *trp*, *Hpa*II in *lac*UV5). *Taq*I and *Hpa*II sites shown in brackets are lost in the *tac* promoter. Circled bases indicate startpoints of transcription (Siebenlist et al., 1980; Rosenberg and Court, 1979). Boxed areas indicate −35 and −10 promoter regions, and the distances between them are specified in bp, just below the sequences.

TCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGCT
TaqI

GTGGTATGGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAA

GGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTT

-35

CTGGCAAATATTCTGAAATGAGCTGTTGACAATTAATCATCGGCTCGTAT
TaqI/HpaII
Hybrid

Pribnow SD

AATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGAATTC
RI

Fig. 4. Nucleotide sequence of the 192-bp *Taq*I −35 *trp* promoter fragment fused to the 55-bp *Hpa*II-*Pvu*II −10 *lac*UV5 promoter fragment. Plasmids ptac11 (Fig. 1) and ptac12 (Fig. 2) served as the source of DNA for the sequencing. In each case, DNA was labeled at the *Eco*RI site and sequencing was according to the method of Maxam and Gilbert (1980). The fusion points with the pBR322 DNA sequences are indicated by arrows. Pribnow: −10 promoter region; SD, RBS; RI, *Eco*RI site.

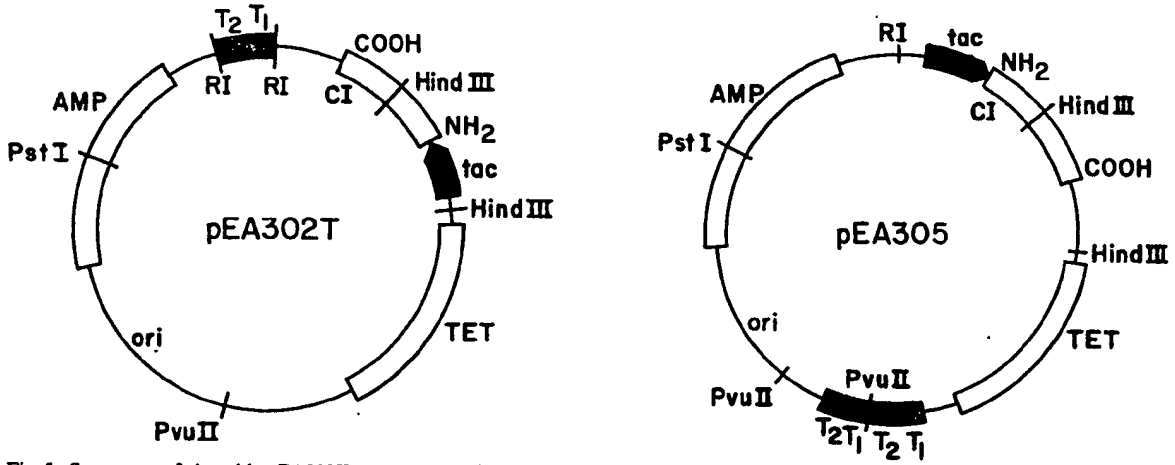


Fig. 5. Structures of plasmids pEA302T and pEA305 (for construction see RESULTS, section e). Both plasmids carry a 1100-bp *Hpa*II fragment inserted downstream of the *tac* promoter which carries a hybrid ribosome binding site with a spacing of 8 bp between the RBS sequence of *lacZ* and the *cI* ATG as follows:

AGGAAACAGCGTATG
RBS cI

To compare the *cI* polypeptide levels made under the control of *tac* and *lac*UV5 promoters, we constructed pEA306 (see RESULTS, section c) which differs from pEA305 only in that the *lac*UV5 promoter replaces the *tac* promoter to drive the synthesis of *cI* protein. The hybrid ribosome binding sites in these two plasmids are identical. For other details see Figs. 1 and 2.

compare directly expression levels of the *c*I gene under control of *tac* and *lac*UV5 promoters, we transferred an *Eco*RI-*Bam*HI fragment of pKB280, containing the *lac*UV5 promoter and the entire *c*I gene, to the *Eco*RI-*Bam*HI backbone of plasmid pKK84-1 yielding pEA306. Plasmids pEA305 and pEA306 are identical except for their different promoters (*tac* in pEA305, *lac*UV5 in pEA306). Therefore, any quantitative difference in *c*I protein production should be due to a difference in promoter strength.

(d) Inducibility and comparison of promoter strength of *tac* and *lac*UV5

Compared to plasmid pEA306, plasmid pEA305 directs the synthesis of five-fold higher levels of *c*I protein upon induction with IPTG. 2 h after induc-

TABLE I

Time course of induction of λ repressor in strain W3110 *lacI*^{Q}L8

Time after induction (min)	Repressor level [a]		
	Promoter		
	tac	*lac*UV5	Ratio *tac*:*lac*UV5
0	< 1	<1	–
15	6	2	3.0
30	12	2	6.0
45	16	3	5.3
60	19	4	4.7
120	26	5	5.2
240	29	6	4.8

[a] The gel presented in Fig. 7 was scanned as described in MATERIALS AND METHODS, section f, and the amount of repressor at each timepoint is specified as % of total cellular protein.

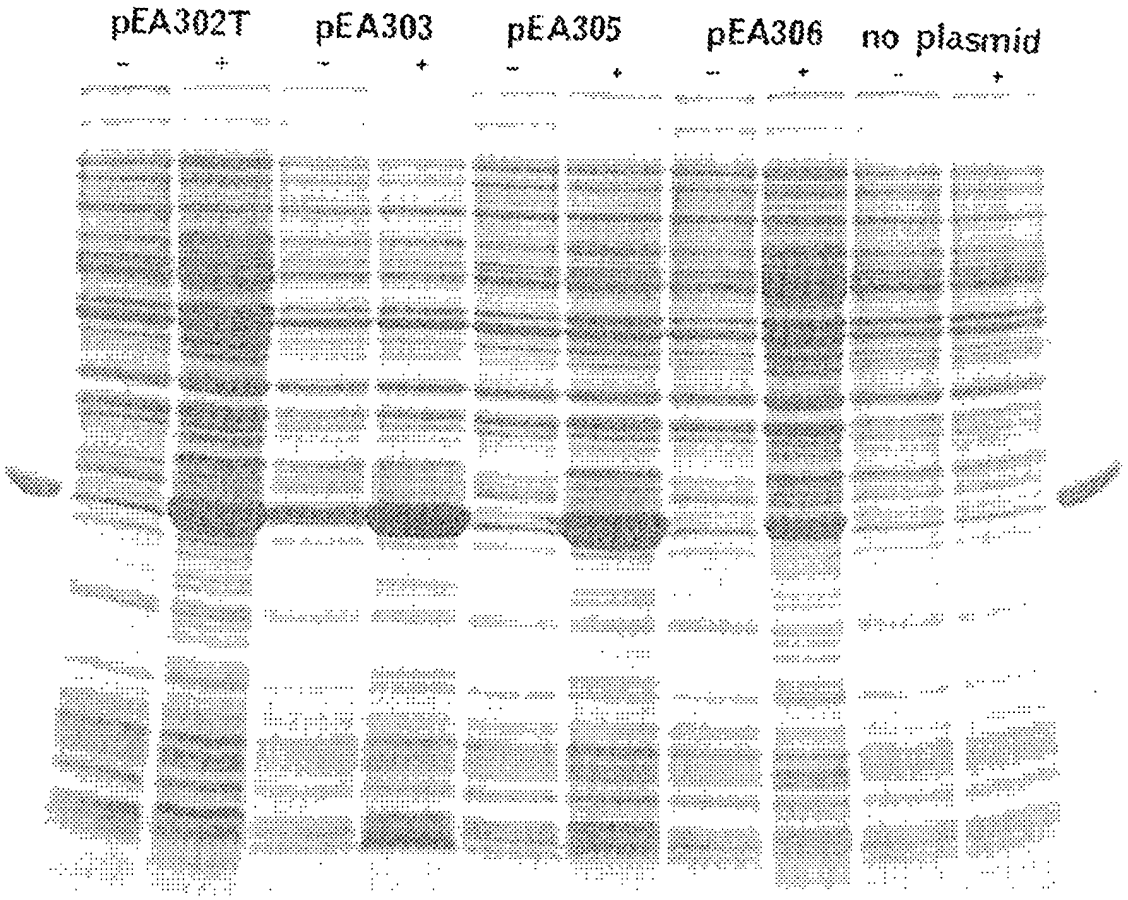


Fig. 6. Induction of *c*I repressor in strain W3110 *lacI*^{Q}L8 carrying *tac* promoter-*c*I and *lac*UV5 promoter-*c*I fusion plasmids. Freshly transformed single colonies were used to inoculate cultures in LB medium in the presence of 50 μg/ml amp. At A_{600} = 0.7 IPTG was added to a final concentration of 1 mM One ml samples were withdrawn immediately, cells centrifuged and frozen at −18°C. The induced cultures were incubated for 2 h, 1 ml samples withdrawn and treated as described in MATERIALS AND METHODS, section f, and analyzed on a 13.5% SDS-polyacrylamide gel. Purified *c*I protein was run in the flanking lanes as a control; "−" indicates uninduced cultures and "+" specifies induced cultures.

tion, yields of *c*I protein were 5% and 26% of total cellular protein (Fig. 6 and Tables I and II). Fig. 7 presents a time course of *c*I protein synthesis by plasmids pEA305 and pEA306 after induction with IPTG. The corresponding amounts of *c*I protein given as percent of total cellular protein are shown in Table I. From these results we conclude that the *tac* promoter is at least five-fold stronger than the *lac*UV5 promoter (see DISCUSSION).

(e) Effects of the relative orientation of the *tac*-promoter-*c*I gene insert on expression levels of *c*I protein

Plasmids pEA302 and pEA305 differ in the relative orientation of the *tac* promoter-*c*I gene insert and pEA305 carries two copies of the *rrnB* transcrip-

TABLE II

Synthesis of lambda repressor in strain W3110 *lacI*^{Q}L8

Plasmid	Promoter	Orientation [a]	Induction	Amount of repressor [b]
pEA302T	*tac*	cc	–	< 1
			+	18
pEA303	*tac*	c	–	8
			+	32
pEA305	*tac*	c	–	< 1
			+	26
pEA306	*lac*UV5	c	–	< 1
			+	5

[a] cc, counterclockwise; c, clockwise

[b] The gel presented in Fig. 6 was scanned as described in MATERIALS AND METHODS, section f. Amount of repressor is given as % of total cellular protein.

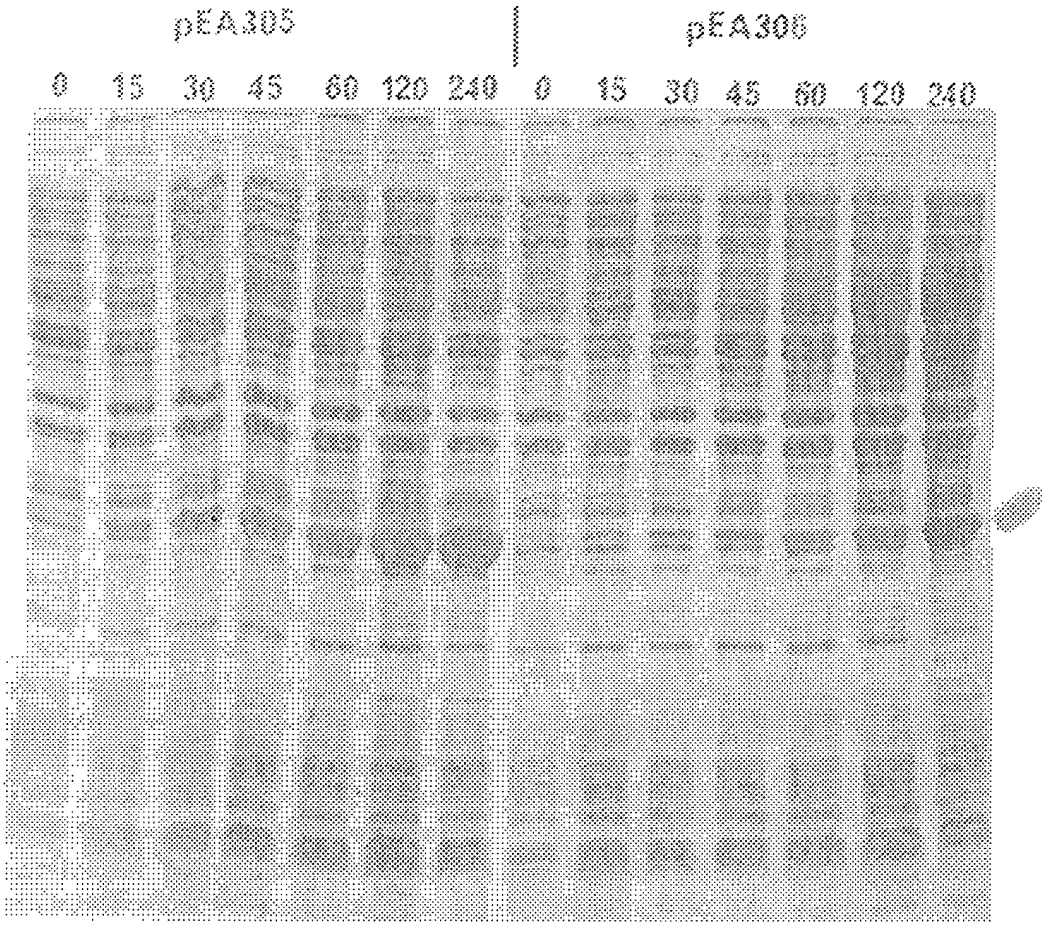


Fig. 7. De novo synthesis of repressor protein upon induction with IPTG of the *tac* promoter (pEA305) or the *lac*UV5 promoter (pEA306) in strain W3110*lacI*^{Q}L8. Cultures were grown as described in legend to Fig. 6. At A_{600} = 0.7, IPTG was added to a final concentration of 1 mM. At the indicated times (in min) after IPTG addition, 1 ml samples were withdrawn and treated as described in legend to Fig. 6. Samples were analyzed on a 13.5% polyacrylamide gel. Synthesis of *c*I protein reaches a plateau 240 min after induction (not shown). Purified *c*I protein was run as a control.

tion terminators (Brosius et al., 1981) while pEA302 does not. To study only the effect of the *tac* promoter-*c*I gene insert orientation on *c*I protein expression levels, we inserted a 500-bp fragment carrying the two transcriptional terminators of the *rrnB* operon into pEA302 downstream from the *c*I gene, thus yielding pEA302T (Fig. 5). Plasmids pEA302T and pEA305 now both carry strong transcriptional terminators downstream of the *c*I gene. Experiments using these two plasmids revealed that *c*I gene expression levels (Fig. 6 and Table II) were higher in the clockwise orientation (18% of total cellular protein for pEA302T as compared to 26% for pEA305). No difference in copy number for these two plasmids could be detected as estimated on agarose gel electrophoresis following the Birnboim and Doly (1979) plasmid-extraction procedure.

(f) Repressibility of the *tac* promoter in *lacI* overproducing strains

Plasmid pEA303, which carries two copies of the *c*I gene but only one *tac* promoter in front of the first *c*I gene (see above) gave the highest expression levels (32% of total cellular protein) of all *tac* promoter-*c*I fusion plasmids (Fig. 6, Table II). In contrast to other *tac* promoter-*c*I gene fusion plasmids with pEA303 in strain W3110*lacI*^{Q}L8, we detected expression of the *c*I gene without IPTG induction (8% of total cellular protein) (Fig. 6, Table II). However, if pEA303 is transformed into strain X90 which carries the stronger *lacI*^{Q1} allele on an F′ episome, no detectable *c*I gene expression was observed (data not shown). In contrast to all other *tac* promoter-*c*I fusion plasmids, pEA303 transforms at a 1000-fold reduced efficiency into strain MM294, which has a *lacI*$^{+}$ allele (data not shown).

If a DNA fragment, which carries the *lacI*^{Q} operon, is cloned into a non-essential region of pEA303, the transformation efficiency is restored to control levels. *tac* promoter-*c*I fusion plasmids carrying the *lacI*^{Q} fragment can be stably maintained in strain LG90, which is deleted for the *lacI* allele, whereas the same plasmid without that fragment cannot transform strain LG90 (data not shown).

These results suggest that high expression levels of *c*I protein are deleterious to the cell, but the presence of the *tac* promoter itself is not since ptac11 and ptac12 are both stably maintained in MM294.

DISCUSSION

Our *tac*-promoter-bearing vectors carry a 192-bp fragment containing sequence from the *E. coli trp* promoter region and a 55-bp fragment carrying sequences from the *lac*UV5 promoter region in the pBR322 derived plasmids. The complete DNA sequences of the vectors are known. The vectors are designed to be generally applicable, and can be used for:

(*i*) insertion of DNA fragments carrying genes to be expressed into unique restriction sites downstream of the *tac* promoter and the *lacZ* RBS sequence (i.e. *Eco*RI in ptac11; *Pvu*II in ptac12; *Hin*dIII in ptac12H).

(*ii*) isolation of "portable promoter fragments" carrying both the *tac* promoter and the *lacZ* RBS sequence to constitute a "hybrid ribosome binding site" (Backman and Ptashne, 1978) when fused to a given gene (for example, the 270-bp *Eco*RI-*Pvu*II fragment from ptac12).

(*iii*) conversion of existing *lac*UV5 promoter gene fusions into more efficiently expressed *tac* promoter-gene fusions using the intermediate plasmids pEA300 and pEA301.

The latter approach has been used here to construct plasmids that express *c*I protein under *tac* promoter control. In identical plasmid constructions (*tac* or *lac*UV5 promoting *c*I protein synthesis), the *tac* promoter directs the synthesis of at least five-fold more repressor protein than the *lac*UV5 promoter (for these plasmids, no difference in copy number was observed, as judged from agarose gel electrophoresis). This finding is likely to represent an underestimate of the relative strength of the *tac* promoter, as the gels are somewhat overloaded for *c*I and gel-scanning cannot quantitate the overloading effect due to proteins being present in high amounts (see Figs. 6 and 7). Also, there may be a nonlinear relationship between accumulation of mRNA and gene product. DeBoer et al. (1983) find that the *tac* promoter is 5–10 times more efficient than the *lac*UV5 promoter for the production of human growth hormone. The amount of human growth hormone per cell was considerably less than that of *c*I protein reported here, which suggests that in our experiments the availability of ribosomes to translate *c*I mRNA may be a limiting factor.

Using the same approach as described above, we

converted the *lac*UV5 promoter of plasmid pLG117R, which directs the synthesis of human fibroblast interferon in *E. coli* (Taniguchi et al., 1980), into a *tac* promoter. The *tac* promoter-interferon fusion plasmid produces 5–10-fold higher levels of mature interferon than does pLG117R (N. Irwin and E. Amann, unpublished results).

Induction of the *tac* promoter was achieved by the addition of IPTG to a mid-log culture and continued incubation. For the *tac*-cI gene fusion plasmids, IPTG concentration of 10^{-4} M gave maximal induction while 10^{-5} M gave no detectable induction as measured by cI gel electrophoresis. Due to its repressibility and inducibility, the *tac* promoter system is useful for the production of proteins which are toxic to *E. coli* or which interfere with normal cell metabolism.

Since the *tac* promoter carries the *lac* operator, its activity is regulated by the *lac* repressor. Activity of the *lac* promoter on a multicopy plasmid in the $lacI^+$ strain MM294 is constitutive (Backman et al., 1976). Our *tac* promoter-cI gene fusion plasmids are also derepressed in strain MM294 (see RESULTS, section f). Without induction, however, there was significant expression of the cI gene in our standard strain W3110$lacI^Q$L8 only if two copies of the *lac* operator sequence were present on the plasmid (pEA303; see RESULTS, section f). If, however, a DNA fragment containing the $lacI^Q$ operon was cloned onto pEA303, this basal level of expression was no longer observed (data not shown). In accordance with this finding is the fact that with the $lacI^Q$ fragment cloned onto it, the plasmid now transforms at normal efficiencies into a $lacI^+$ strain (MM294) or into a *lacI* deletion strain (LG90) (not shown). Thus, by introducing the "portable $lacI^Q$ fragment" from plasmid p$lacI^Q$ into any *tac* promoter-gene fusion plasmid, the resulting hybrid plasmid becomes autorepressable and can be transformed into any *E. coli* strain.

Our *tac* promoter vectors are currently in use in several laboratories for expression of various prokaryotic and eukaryotic polypeptides. These include wild-type and mutant phage repressor proteins (λ, 434, P22 repressor and *cro* proteins); P22 *erf* protein (A. Poteete and A. Fenton, submitted); *lexA* protein (R. Brent, unpublished); *uvr* proteins (A. Sancar, submitted); *mal* proteins (H. Shuman and E. Amann, in preparation).

Eukaryotic proteins that are expressed include rat and human insulin (S. Stahl, J. Brosius, I. Akerblom and W. Gilbert, unpublished); human serum albumin (B. Wallner-Philipp, personal communication); human leukocyte interferon (J. Brosius, K. Henco, S. Nagata, C. Weissmann, G. Avgerinos, P. Levine, W. Gilbert, unpublished); human fibroblast interferon (N. Irwin and E. Amann, unpublished); SV40 small tumor antigen (Bikel et al., 1983); SV40 large T antigen (K. Zinn and E. Amann, unpublished).

In all cases where there is a comparison with similar *lac* promoter-gene fusions possible, 5–10-fold higher expression levels are observed with the *tac* promoter.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the NIH (1RO1 AI1858o1) to Mark Ptashne and in part by a grant from Biogen S.A. to Walter Gilbert. E. Amann and J. Brosius were supported by fellowships of the Deutsche Forschungsgemeinschaft. J. Brosius thanks Water Gilbert for laboratory space, support and discussions. We would like to thank the members of the Ptashne group for numerous suggestions, discussions and critical reading of the manuscript.

REFERENCES

Backman, K., Ptashne, M. and Gilbert, W.: Construction of plasmids carrying the cI gene of bacteriophage lambda. Proc. Natl. Acad. Sci. USA 73 (1976) 4174–4178.

Backman, K. and Ptashne, M.: Maximizing gene expression on a plasmid using recombination in vitro. Cell 13 (1978) 65–71.

Bennett, G.N., Schweinegruber, M.E., Brown, K.D., Squires, C. and Yanofski, C.: Nucleotide sequence of the promoter-operator region of the trypthophan operon of *E. coli*. J. Mol. Biol. 121 (1978) 113–137.

Bikel, J., Roberts, T.A., Bladon, M.T., Green, R., Amann, E. and Livingstone, D.M.: Purification of biologically active simian virus 40 small tumor antigen. Proc. Natl. Acad. Sci. USA 80 (1983) 906–910.

Birnboim, H.C. and Doly, J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7 (1979) 1513–1523.

Blattner, F.R., Fiandt, M., Hass, K.K., Twose, P.A. and Szybalski, W.: Deletion and insertions in the immunity region of coliphage lambda: Revised measurement of the promoter-startpoint distance. Virology 62 (1974) 458–471.

Brent, R. and Ptashne, M.: Mechanism of action of the *lexA* gene product. Proc. Natl. Acad. Sci. USA 78 (1981) 4204–4208.

Brosius, J., Dull, T.J., Sleeter, D.D. and Noller, H.F.: Gene organization and primary structure of a ribosomal RNA operon from *E. coli*. J. Mol. Biol. 148 (1981) 107–127.

deBoer, H.A., Comstock, L.J., Yansura, D.G. and Heynecker, H.L.: Construction of a tandem *trp-lac* promoter and a hybrid *trp-lac* promoter for efficient and controlled expression of the human growth hormone gene in *Escherichia coli*, in Rodriguez, R.L. and Chamberlin, M.J. (Eds.), Promoters, Structure and Function. Praeger, New York, 1982, pp. 462–481.

deBoer, H.A., Comstock, L.J. and Vasser, M.: The *tac* promoter: A functional hybrid derived from *trp* and *lac* promoters. Proc. Natl. Acad. Sci. USA 80 (1983) 21–25.

Gentz, R., Langner, A., Chang, A.C.Y., Cohen, S.N. and Bujard, H.: Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. Proc. Natl. Acad. Sci. USA 78 (1981) 4936–4940.

Gilbert, W.: Starting and stopping sequences for the RNA polymerase, in Losick, R. and Chamberlin, M. (Eds.), RNA Polymerase. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1976, pp. 193–205.

Guarente, L., Lauer, G., Roberts, T.M. and Ptashne, M.: Improved methods for maximizing expression of a cloned gene: a bacterium that synthesizes rabbit β-globin. Cell 20 (1980) 543–553.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680–685.

Lauer, G., Pastrana, R., Sherley, J. and Ptashne, M.: Construction of overproducers of the bacteriophage 434 repressor and *cro* proteins. J. Mol. Appl. Genet. 1 (1981) 139–147.

Mandecki, W. and Reznikoff, W.S.: A *lac* promoter with a changed distance between −10 and −35 regions. Nucl. Acids Res. 10 (1982) 903–912.

Mandel, M. and Higa, A.: Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 53 (1970) 159–162.

Maniatis, T., Ptashne, M., Backman, K., Kleid, D., Flashman, S., Jeffrey, A. and Maurer, R.: Recognition sequences of repressor and polymerase in the operators of bacteriophage lambda. Cell 5 (1975) 109–113.

Maxam, A.M. and Gilbert, W.: Sequencing end-labeled DNA with base-specific chemical cleavages, in Grossman, L. and Moldave, K. (Eds.), Methods in Enzymology, Vol. 65, Academic Press, New York, 1980, pp. 499–560.

Pribnow, D.: Nucleotide sequence of an RNA polymerase binding site at an early T7 promotor. Proc. Natl. Acad. Sci. USA 79 (1975) 1069–1072.

Rosenberg, M. and Court, D.: Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. 13 (1979) 319–353.

Russell, D.R. and Bennett, G.R.: Construction and analysis of in vivo activity of *E. coli* promoter hybrids and promoter mutants that alter the −35 to −10 spacing. Gene 20 (1982) 231–243.

Schaller, H., Gray, C. and Herrmann, K.: Nucleotide sequence of an RNA polymerase binding site from the DNA of bacteriophage fd. Proc. Natl. Acad. Sci. USA 72 (1975) 737–741.

Siebenlist, U., Simpson, R.B. and Gilbert, W.: *E. coli* RNA polymerase interacts homologously with two different promoters. Cell 20 (1980) 269–281.

Stefano, J.E. and Gralla, J.D.: Spacer mutations in the *lac* p^s promoter. Proc. Natl. Acad. Sci. USA 79 (1982) 1069–1072.

Sutcliffe, J.G.: Complete nucleotide sequence of the *E. coli* plasmid pBR322. Cold Spring Harbor Symp. Quant. Biol. (1979) 77–90.

Talmadge, K. and Gilbert, W.: Construction of plasmid vectors with unique *Pst*I cloning sites in a signal sequence coding region. Gene 12 (1980) 235–241.

Taniguchi, T., Guarente, L., Roberts, T.M., Kimelman, D., Douhan III, J. and Ptashne, M.: Expression of the human fibroblast interferon gene in *E. coli*. Proc. Natl. Acad. Sci. USA 77 (1980) 5230–5233.

Youderian, P., Bouvier, S. and Susskind, M.M.: Sequence determinants of promoter activity. Cell 30 (1982) 843–853.

Communicated by G.A. Wilson.

JOURNAL OF BACTERIOLOGY, July 1989, p. 3760–3766
0021-9193/89/073760-07$02.00/0

Vol. 171, No. 7

DnaA Protein Overproduction Abolishes Cell Cycle Specificity of DNA Replication from *oriC* in *Escherichia coli*

OLGA PIERUCCI,* MICHAEL RICKERT, AND CHARLES E. HELMSTETTER†

Department of Experimental Biology, Roswell Park Memorial Institute, Buffalo, New York 14263

Received 9 December 1988/Accepted 3 April 1989

Initiation of DNA replication from *oriC* in *Escherichia coli* takes place at a specific time in the cell division cycle, whether the origin is located on a chromosome or a minichromosome, and requires participation of the product of the *dnaA* gene. The effects of overproduction of DnaA protein on the cell cycle specificity of the initiation event were determined by using minichromosome replication as the assay system. DnaA protein was overproduced by inducing the expression of plasmid-encoded *dnaA* genes under control of either the p*tac* or lambda p_L promoter. Induction of DnaA protein synthesis caused a burst of minichromosome replication in cells at all ages in the division cycle. The magnitude of the burst was consistent with the initiation of one round of replication per minichromosome in all cells. The replication burst was followed by a period of reduced minichromosome replication, with the reduction being greater at 30 than at 41°C. The results support the idea that the DnaA protein participates in *oriC* replication at a stage that is limiting for initiation. Excess DnaA protein enabled all cells to achieve the state required for initiation of DNA polymerization by either effecting or overriding the normal limiting process.

In *Escherichia coli*, DNA synthesis initiates at the chromosomal origin of replication, *oriC*, at a specific time during the division cycle (14–17, 20). The biochemical process responsible for this timing mechanism has not been identified, but it has been suggested that the product of the *dnaA* gene, which is required for initiation of replication from *oriC*, could be involved (3, 7, 24, 30, 39). The DnaA protein binds cooperatively to several sites (DnaA boxes) located within *oriC* (10, 13, 22). In so doing, it participates in the assembly of the complex of initiation proteins that eventually leads to the unwinding, priming, and polymerization of the *oriC* region (4, 11, 36). The DnaA protein also binds to DnaA boxes within the promoter of the *dnaA* gene itself (1, 8, 19, 40), the *mioC* gene adjacent to *oriC* (21, 31, 33), and several other genes, some of which are involved in DNA metabolism (10). Thus, in addition to its direct role in initiation of replication at *oriC*, the DnaA protein may also act to regulate transcription of a number of DNA synthesis-related genes (23).

When the intracellular quantity of DnaA protein is increased, through induction of synthesis from plasmid-encoded *dnaA* genes, replication from *oriC* is stimulated (2). This is the case whether *oriC* is located on the chromosome or on an extrachromosomal plasmid (a minichromosome). Replication of the minichromosomes progresses to completion. The newly initiated chromosomal replication forks are stalled shortly after initiation (2, 41), although addition of rifampin appears to enable the stalled forks to proceed along the chromosome (28). These findings suggest that overproduction of DnaA protein could alter the normal timing of initiation of DNA replication from *oriC* during the division cycle and, as a result, cause initiation events at earlier-than-normal times in the cycle. To examine this issue, we determined the kinetics of minichromosome replication during overproduction of DnaA protein at various times during the division cycle of synchronously growing *E. coli* B/r F (15). It was found that minichromosome replication could be induced in cells of all ages.

MATERIALS AND METHODS

Bacteria and plasmids. Experiments were performed with *E. coli* B/r F *thyA his* (15) harboring the minichromosome pAL49 or pAL4 (20). The *oriC* region of pAL49, but not that of pAL4, contains the *mioC* gene (20). Plasmids pLSK5 and pTTQ9 both harbor the $lacI^q$ repressor gene and the *trp-lac* promoter (p*tac*). The p*tac* promoter is upstream of the *dnaA* gene in pLSK5 and upstream of the beta-galactosidase alpha-fragment gene in pTTQ9 (38). Plasmid pLSK5 was obtained from W. Messer, via J. Zyskind. Plasmid pTAC1445 carries the *dnaA* structural gene under the control of the lambda p_L promoter (2). In plasmid pTAC1584, a derivative of plasmid pTAC1445, the *dnaA* gene contains a 121-base-pair deletion and produces a nonfunctional DnaA protein (2). Plasmid pALO8 contains the cI857 gene, encoding the thermolabile lambda repressor protein (2). Induction of plasmid-coded *dnaA* gene expression was obtained in *E. coli* B/r F(pLSK5) by exposure of the cells to the inducer isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 10^{-3} M) and in B/r F(pALO8, pTAC1445) by a temperature shift from the growth temperature, 30°C to 41°C, the temperature of inactivation of the lambda repressor protein.

Growth media and chemicals. Cells were grown at 30°C in minimal salts medium (15) supplemented with thymine (10 μg/ml), histidine (20 μg/ml), and methionine (20 μg/ml). Glucose (0.2%) was used as the carbon source. Ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), kanamycin (100 μg/ml), and tetracycline (10 μg/ml) were present for plasmid-containing cells, as necessary. IPTG, ampicillin (sodium salt), and tetracycline hydrochloride were from Sigma Chemical Co., St. Louis, Mo. Chloramphenicol and kanamycin sulfate were from United States Biochemical Corp., Cleveland, Ohio. Rifampin was from Boehringer GmbH, Mannheim, Federal Republic of Germany. Plasmid pTTQ9 was from Amersham Corp., Arlington Heights, Ill.

Minichromosome replication. The rates of DNA replication were measured in samples (1 or 2 ml) of cultures exposed to

* Corresponding author.
† Present address: Department of Biological Sciences, Florida Institute of Technology, Melbourne, FL 32901.

[*methyl*-^{3}H]thymidine (10 μCi/ml; specific activity, 80 Ci/mmol) for 4 or 5 min. For both minichromosomes and chromosomes, the relative changes in DNA content were determined in samples (2 ml) of cultures continuously exposed to [*methyl*-^{3}H]thymidine (8 μCi/ml; specific activity, 60 mCi/mmol). Radioactivity incorporation was stopped by the addition of excess nonradioactive thymidine (final concentration, 200 μg/ml) for 10 min. A portion (0.1 or 0.2 ml) of each sample was placed in ice-cold, 5% trichloroacetic acid (TCA) for estimation of the total cellular radioactivity incorporation. The remaining portion was used for the analysis of minichromosome radioactivity incorporation by the procedure described by Projan et al. (29). Twenty microliters of whole-cell lysate (total volume, 50 or 80 μl) was loaded into each well of a 0.7% horizontal agarose gel. Electrophoresis in Tris borate-EDTA buffer was performed for 18 h at 40 V. Gels were prepared for fluorography as described previously (28), and the dried gels were exposed to X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) at −70°C. The autoradiographs of the minichromosome and plasmid DNA bands were scanned with a video densitometer (Bio-Rad Laboratories, Richmond, Calif.).

Cell cycle analysis during short periods of DnaA protein overproduction. The effects of short periods of enhanced *dnaA* gene expression on the initiation of DNA replication were analyzed in multiple samples of synchronously growing cells obtained as previously described (15, 20). Briefly, overnight cultures grown at 30°C in media supplemented with the appropriate antibiotics were diluted (1:200) in 100 ml of medium lacking the antibiotics and grown at the same temperature for at least four mass doublings. At a cell concentration of 10^8 cells per ml, the cultures were filtered onto the surface of a type GS Millipore nitrocellulose filter and washed with 100 ml of minimal medium, and the filters were inverted. Newborn cells were eluted at 30°C; 18 consecutive samples taken at 2-min intervals were collected, and portions (5 ml) were grown synchronously at 30°C for different time periods, namely 90, 85, . . . 10, and 5 min. After these periods, one-half of each sample was exposed to IPTG for 7 or 10 min. During the last 5 min of the induction period, 2 ml of the induced and uninduced portions of each synchronous sample was exposed to [*methyl*-^{3}H]thymidine (10 μCi/ml). Cell concentration was determined both at the time of collection and 1 min prior to the radiolabeling.

Cell cycle analysis during continuous DnaA protein overproduction. The effect of long-term induction of *dnaA* gene expression on minichromosome replication was analyzed in cells of specific ages in the cycle. Twenty consecutive samples of newborn cells taken at 1-min intervals were collected, and a 4-ml portion of each sample was grown at 30°C for either 0 (age 0) or 35 (age 0.65) min. IPTG was added to one-half of each sample of the chosen cell age, and growth was continued thereafter for different time periods, namely 96, 91, . . . 6, and 1 min. At these times the samples of both the induced and uninduced cells were exposed to [*methyl*-^{3}H]thymidine for 5 min.

The rates of minichromosome replication during the division cycle were estimated as the ratio of the intensity of the minichromosome DNA bands obtained from synchronous cells to the cell number at the time of collection. The doubling times were calculated as the times required for the cell concentrations to increase 1.5-fold during the synchronous growth. The cell ages at the time of exposure to radioactive thymidine were computed as the ratios of the midtimes of the labeling periods during synchronous growth to the doubling times of the synchronous cultures.

Absorbance, cell concentration, and radioactivity. The A_{450} of cultures was determined with a Zeiss PMQII spectrophotometer. Cell concentrations were determined with a Coulter Counter (model ZB; Coulter Electronics, Inc., Hialeah, Fla.). [*methyl*-^{3}H]thymidine (78 to 80 Ci/mmol) was purchased from ICN Radiochemicals, Irvine, Calif. Incorporation of [^{3}H]thymidine into cellular DNA was measured by cold 5% TCA-precipitated radioactivity retained on filter disks. The radioactivity in samples was counted in Spectrafluor (Amersham) with a liquid scintillation counter (LS-7000; Beckman Instruments, Inc., Fullerton, Calif.).

RESULTS

Minichromosome replication upon induction of plasmid-encoded *dnaA* gene expression. Minichromosome replication was analyzed during DnaA protein overproduction in steady-state cultures of *E. coli* B/r F containing the *oriC* plasmid pAL49 or pAL4. DnaA protein overproduction was achieved in minichromosome-containing cells in two ways: (i) by the addition of IPTG to cells harboring pLSK5 or (ii) by thermoinduction of cells harboring pTAC1445. The kinetics of minichromosome replication were determined by pulse-labeling samples with [^{3}H]thymidine at various times after induction of *dnaA* gene expression and visualizing the incorporation by agarose gel electrophoresis of whole-cell lysates.

The radioactivity in pAL49 minichromosome bands at consecutive 2-min intervals following induction of *dnaA* gene expression from plasmid pLSK5 by IPTG addition is shown in Fig. 1, row a. Overproduction of the DnaA protein resulted in a burst of minichromosome replication. An increase in the rate of pAL49 replication was not seen when IPTG was added to cells containing plasmid pTTQ9, which encodes the beta-galactosidase alpha-fragment gene in place of the *dnaA* structural gene (row b). The effects of thermoinduction of DnaA protein synthesis in *E. coli* B/r F(pALO8, pTAC1445) containing pAL49 or pAL4 are shown in rows c and f, respectively. Replication was enhanced immediately for both minichromosomes. The enhancement was absent in B/r F(pALO8, pTAC1584), in which the DnaA protein was defective (rows d and g), and B/r F, which did not contain an inducible *dnaA* gene (rows e and h). However, a transient inhibition of minichromosome replication was associated with the temperature shift from 30 to 41°C in cells in which a functional DnaA protein was not induced.

The enhanced minichromosome replication resulting from overproduction of DnaA protein was followed by a period of reduced replication (Fig. 1, rows a, c, and f). The rate of replication during this period was higher when the DnaA overproduction was induced by a shift from 30 to 41°C (rows c and f) than when the temperature remained at 30°C (row a). This higher level of subsequent replication was a consequence of the temperature upshift; indeed, when DnaA protein synthesis was induced by IPTG in B/r F(pLSK5) concomitant with an upshift in temperature (row i), the rate of minichromosome replication was similar to that observed following thermoinduction of DnaA protein synthesis. Again, the temperature shift alone in the absence of induced DnaA protein synthesis was associated with a temporary inhibition of minichromosome replication (row j).

The kinetics of pAL49 minichromosome replication in selected experiments from Fig. 1 are shown in quantitative form in Fig. 2. The absorbances of the DNA bands in Fig. 1, row a [B/r F(pLSK5)] and row b [B/r F(pTTQ9)], upon induction of DnaA protein and the beta-galactosidase alpha

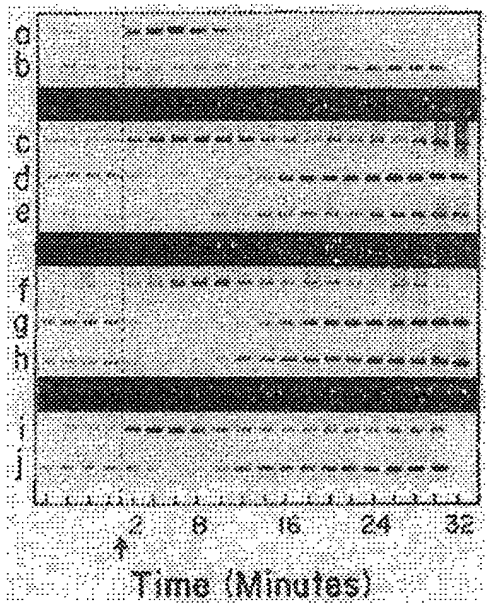


FIG. 1. Minichromosome replication during overproduction of the DnaA protein: fluorographs of minichromosome DNA bands. Steady-state cultures of *E. coli* B/r F harboring the minichromosome pAL49 or pAL4 were grown under selective pressure at 30°C. At a cell concentration of about 5×10^7 cells per ml, cells carrying the pLSK5-coded *dnaA* gene were induced by IPTG and cells harboring the pTAC1445-coded *dnaA* gene were thermoinduced by a temperature shift from 30 to 41°C. At various times before and after DnaA induction, samples (1 ml) were radioactively labeled with 10 μCi of [^{3}H]thymidine per ml for 4 min. After lysis, electrophoresis, and fluorography, the minichromosome DNA bands were photographed. Rows: a and b, pAL49 minichromosome DNA bands during exposure to IPTG at 30°C [a, B/r F(pLSK5); b, B/r F(pTTQ9)]; c, d, and e, pAL49 minichromosome DNA bands after a temperature shift from 30 to 41°C [c, B/r F(pALO8, pTAC1445); d, B/r F(pALO8, pTAC1584); e, B/r F]; f, g, and h, pAL4 minichromosome DNA bands after a temperature shift from 30 to 41°C [f, B/r F(pALO8, pTAC1445); g, B/r F(pALO8, pTAC1584); h, B/r F]; i and j, pAL49 minichromosome DNA bands from B/r F(pLSK5) after a temperature shift from 30 to 41°C [i, with IPTG; j, without IPTG]. The broken vertical line indicates the time of IPTG addition and/or the temperature shift. The midpoints of the periods of radioactive-thymidine labeling are indicated at the bottom.

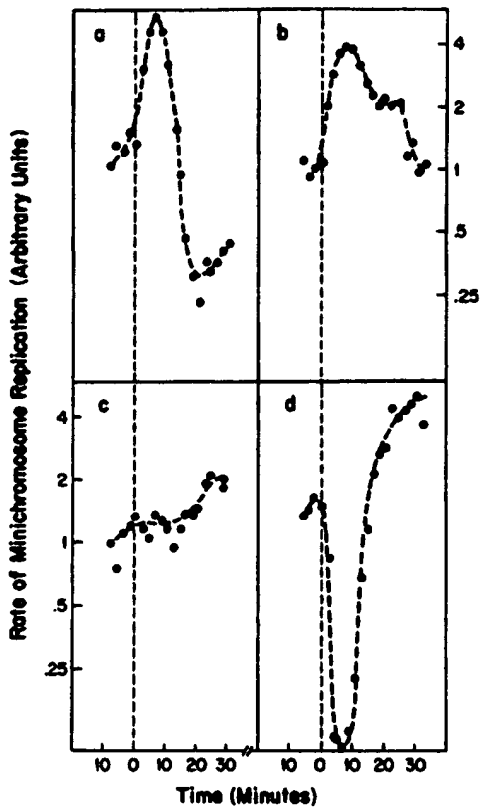


FIG. 2. pAL49 minichromosome replication during induction of *dnaA* gene expression. The intensities of the pAL49 minichromosome DNA bands from fluorographs such as those shown in Fig. 1 were determined by two-dimensional densitometry and plotted as a function of time after the induction of DnaA protein synthesis. Shown are the rates of pAL49 minichromosome replication in B/r F(pLSK5) (a) and B/r F(pTTQ9) (c) upon IPTG induction at 30°C and in B/r F(pALO8, pTAC1445) (b) and B/r F(pALO8, pTAC1584) (d) upon a shift in temperature from 30 to 41°C. The broken vertical lines indicate the time of IPTG addition or the temperature shift.

fragment at 30°C are shown in Fig. 2a and c, respectively. Replication was enhanced approximately fivefold at 6 min after induction of DnaA protein synthesis and was slightly depressed in the absence of induction. Figures 2b and d show the absorbances of the DNA bands in Fig. 1, rows c and d, as a function of time after thermoinduction of B/r F(pALO8, pTAC1445) and B/r F(pALO8, pTAC1584), respectively. In these experiments, the rate of replication was enhanced about 4-fold when functional DnaA protein was produced (Fig. 2b) but transiently depressed about 10-fold when it was not (Fig. 2d).

Chromosome replication in the presence of rifampin. We have previously shown (28) that new, functional replication forks are activated on the chromosome upon thermoinduction of DnaA protein synthesis in B/r F(pTAC1445, pALO8), based on the extent of runout replication in the presence of rifampin. The rapid enhancement of minichromosome replication from plasmid pLSK5 at 30°C upon induction of the *dnaA* gene product with IPTG (Fig. 1 and 2) suggests that new replication forks might be similarly activated on the chromosome, independent of a temperature upshift. To analyze this possibility, steady-state cultures of B/r F(pLSK5) were grown at 30°C for at least 20 generations in the presence of [^{3}H]thymidine to fully label chromosomal DNA and then divided into four portions. One portion of the culture received IPTG at 30°C, while another portion received IPTG and was simultaneously shifted to 41°C. The third and fourth portions were maintained at 30°C, or shifted to 41°C, without the addition of IPTG. After 12 min, rifampin was added to each portion. The radioactivity incorporated into cold TCA-precipitable material was plotted as a function of time after rifampin addition (Fig. 3). In cultures induced with IPTG, the incorporation reached identical plateaus, at 4 h for 30°C and 2 h for 41°C (Fig. 3a). This plateau was at a value about 2.4 times that at the time of rifampin addition, independent of the temperature shift. In uninduced cells (Fig. 3b), incorporation reached maximum values of 1.4 and 1.6 times the initial value at 30 and 41°C, respectively. Thus, induction of the *dnaA* gene product with IPTG was associated with an increased level of chromosomal runout replication, consistent with the activation of functional replication

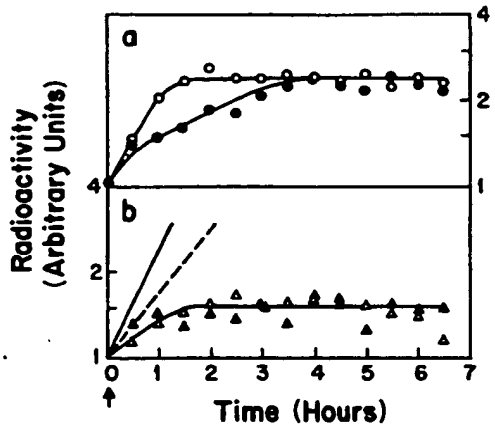


FIG. 3. Runout chromosome replication in the presence of rifampin. A culture of B/r F(pLSK5) was grown for approximately 20 generations in the presence of 8 μCi of [^{3}H]thymidine per ml (specific activity, 60 mCi/mmol) under selective pressure. (a) Portions of the culture received IPTG at 30°C (●) or concomitant with a temperature shift to 41°C (○); (b) portions of the culture were maintained at 30°C (▲) or shifted to 41°C (△) without addition of IPTG. Twelve minutes later, rifampin (final concentration, 100 μg/ml) was added to all the cultures. The radioactivity incorporated into cold 5% TCA-insoluble material at various times after rifampin addition is plotted as a function of time. The arrow indicates the time of rifampin addition. The broken and solid lines in panel b indicate incorporation at 30°C and during a shift from 30 to 41°C, respectively, in the absence of rifampin.

forks on the chromosome, independent of a temperature shift.

Minichromosome replication in synchronously growing *E. coli* B/r F during short periods of DnaA protein overproduction. Minichromosome replication is normally restricted to a discrete interval in the cell division cycle, coincident with initiation of chromosome replication (14). The burst of minichromosome replication observed during induction of DnaA protein synthesis in exponentially growing cultures could be a consequence of enhanced synthesis of minichromosomes during this replication interval or of the activation of replication potential in cells at other (earlier) ages in the division cycle. To discriminate between these possibilities, samples of *E. coli* B/r F(pLSK5) of various ages in the division cycle were divided into two equal portions. In one, synthesis of the *dnaA* gene product was induced for either 7 or 10 min, while the other portion served as an uninduced control. Induced and uninduced portions of each synchronous sample were labeled with [^{3}H]thymidine during the last 5 min of the induction period and processed and analyzed under the same conditions. Figures 4 and 5 show the DNA bands and densitometric scans of the bands, respectively, for the induced and uninduced samples in both experiments. Minichromosome replication in the uninduced cells was periodic, with a maximum value about midway through the division cycle. DnaA protein overproduction stimulated minichromosome replication in cells of all ages. In the induced cells, the rate of replication followed a stepwise pattern, with a doubling shortly after the interval of pAL49 replication in the uninduced culture. The amount of radioactivity in each sample of the induced culture was approximately equal to the total incorporation in all samples from the corresponding cell cycle of the uninduced culture. This is consistent with the initiation of one round of replication per minichromosome upon induction of DnaA protein synthesis, independent of the stage in the cell cycle. Thus, the stepwise doubling in [^{3}H]thymidine incorporation during the cell cycle reflected the average number of minichromosomes present per cell at the time DnaA protein synthesis was induced. Similar enhancement in minichromosome replication was seen in B/r F(pALO8, pTAC1445) during thermoinduction of DnaA protein synthesis (data not shown).

Minichromosome replication in synchronously growing *E. coli* B/r F during continuous DnaA protein overproduction. The preceding information indicates that cells of all ages were stimulated to replicate their minichromosome contents during the initial stages of DnaA overproduction, but the experiments did not indicate whether the kinetics of replication differed in cells of different ages or whether there was more than one burst of replication. To answer these questions, synchronous samples of cells with ages of 0 and 0.65 generations were divided in half and IPTG was added to one portion. Synchronous growth was continued, and portions were pulse-labeled with [^{3}H]thymidine at consecutive intervals. The absorbances of the radioactive minichromosome bands were determined and plotted as a function of cell age after IPTG addition (Fig. 6). In both experiments, overproduction of the DnaA protein was associated with an immediate enhancement in minichromosome replication. The initial burst of minichromosome replication was followed by a period of significantly reduced replication. At about one generation after the first burst, there may have been a slight second burst of replication, but the level of incorporation was too low to accurately assess the extent of replication at this time. Finally, the total radioactivity in the initial peak in the induced culture was approximately equal to the total incorporation in all samples of the uninduced culture in the same cell cycle, again consistent with one replication per minichromosome upon DnaA overproduction.

DISCUSSION

Overproduction of the DnaA protein was associated with an abrupt increase in the rate of minichromosome replication, independent of the presence or absence of the *mioC* gene on the minichromosome. The extent of replication was

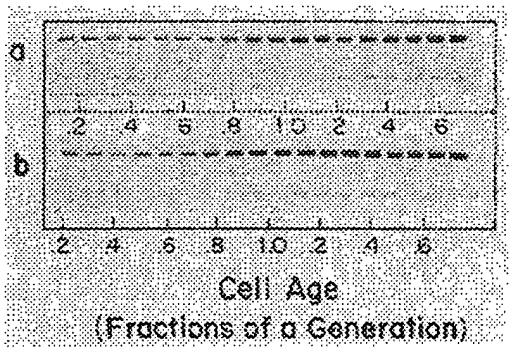


FIG. 4. Minichromosome replication in the division cycle during short periods of DnaA protein overproduction. The fluorographs of the pAL49 minichromosome DNA bands from synchronous B/r F(pLSK5) cells IPTG induced for 7 min (a) and 10 min (b) and radiolabeled for the last 5 min of induction are shown. In each set, the upper row represents the DNA bands of the induced samples and the lower row represents those of the corresponding uninduced samples. The ages of the synchronous cells at the start of induction are indicated at the bottom of each set.

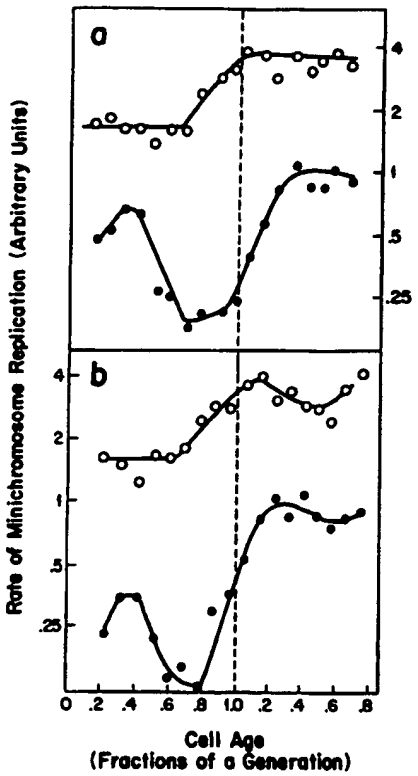


FIG. 5. Rate of minichromosome replication in the cell division cycle during short periods of DnaA protein overproduction. The intensities of the pAL49 minichromosome DNA bands shown in Fig. 4 were normalized to the cell number at the start of synchronous growth and plotted as a function of cell age at the time of radiolabeling. DnaA protein synthesis induction was for 7 min (a) and 10 min (b). Symbols: O, induced cells; ●, uninduced cells. The vertical broken lines indicate the midpoints of synchronous division.

consistent with one round of replication per minichromosome, in agreement with previous findings by Atlung et al. (2). This stimulation of replication took place at all stages in the division cycle. The burst of replication was followed by a period of reduced replication, more evident at 30 than at 41°C, and then about one generation later by a possible second wave of replication at a 10-fold-lower amplitude. Thus, the normal cell cycle specificity of minichromosome replication was overridden by induction of DnaA protein synthesis. If there was any period in the cycle when minichromosome replication could not be induced, such as immediately after replication when the molecules are still hemimethylated (25, 27, 32, 37), it was too short to be detected by these procedures.

The mechanism by which the *dnaA* gene product, when in excess, accelerates the expression of initiation potential can be explained in the following way. The DnaA protein acts early in the initiation reaction (35, 42). According to in vitro studies of minichromosome replication, the active ATP-DnaA form (34) participates in the opening of the DNA duplex at *oriC* (7, 18) to facilitate entry of additional proteins involved in subsequent priming and polymerization steps (4, 6). Overexpression of DnaA protein could induce initiation if an excess of the protein was sufficient to cause formation of the open complex (7) and the other components involved in the later stages of replication were not limiting. This condition would obtain if DnaA was normally limiting for initiation or if the normal limiting step in open complex formation was circumvented by the excess DnaA protein. It has also been suggested that RNA polymerase acts early in initiation and facilitates open complex formation by transcriptional activa-

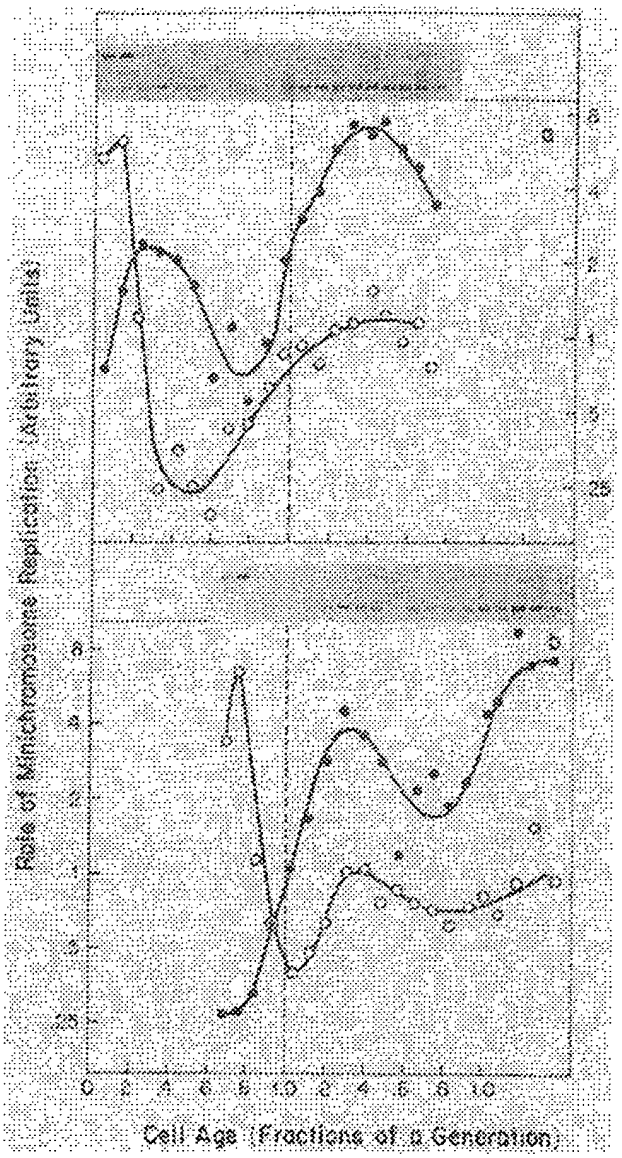


FIG. 6. Rate of minichromosome replication during continuous expression of the *dnaA* gene in the division cycle. Synchronous samples of B/r F(pLSK5) were grown for either 0 min (age 0) (a) or 35 min (age 0.65) (b), and then one-half of each sample received IPTG and the other did not. Growth was continued, and the samples were radioactively labeled at intervals with [^{3}H]thymidine for 5 min. Photographs of the radioactive minichromosome DNA bands are shown at the tops of panels a and b. In each set, the upper row represents the minichromosomes of the induced samples and the lower row represents those of the corresponding uninduced samples. The rate of minichromosome replication was determined as for Fig. 5 and plotted as a function of the age at the time of radiolabeling. The vertical broken lines indicate the times of synchronous division. The rates of minichromosome replication in induced (O) and uninduced (●) cells are shown.

tion at *oriC* (5). The two proteins might thus act to achieve the same topological alteration of the origin region. In fact, initiation of replication from *oriC* is also stimulated by an increase in the availability of RNA polymerase (40a). An elevated ATP-DnaA level could obviate the need for direct polymerase function at the origin. Thus, RNA polymerase action might no longer be needed in the presence of ATP-DnaA in excess, and conversely, initiation might take place in the presence of lower levels of DnaA when RNA polymerase availability is increased.

The period of depressed minichromosome replication after the initial stimulation by DnaA protein could be a reflection of several factors, including modification of DNA topology during overinitiation at *oriC* (6; D. W. Smith and J. W. Zyskind, *in* K. W. Adolph, ed., *Chromosomes: Eucaryotic, Procaryotic and Viral*, vol. 2, in press) or DnaA-mediated effects on the transcription of essential DNA replication-related genes (30). Inhibition of chromosome replication has previously been reported upon IPTG induction of the *dnaA* gene under *ptac* promoter control (31). The inhibition was associated with a decrease in the extent of counterclockwise transcription entering *oriC* during increased DnaA protein synthesis.

The effects of the treatments used for the induction of plasmid-coded *dnaA* gene expression in cells lacking an inducible plasmid-coded *dnaA* gene were also of interest. Temperature upshifts, in the absence of induced DnaA protein synthesis, were associated with a temporary inhibition of minichromosome replication. This early, transient inhibition of replication was specific for the minichromosome; i.e., the rates of replication of plasmids pLSK5, pALO8, pTAC1445, and pTAC1584 all increased immediately upon a shift in temperature from 30 to 41°C. When minichromosome replication resumed, it was at a rate higher than that in unshifted cells. The explanation for this thermal inhibition of minichromosome replication, which was reversed by DnaA protein overproduction, is unknown but could be related to changes in transcriptional patterns (9). Enhanced transcription from heat shock genes (26) could serve to reduce the availability of RNA polymerase for initiation. Excess DnaA protein might serve to overcome the reduced polymerase availability by opening the DNA duplex at *oriC* without the need for transcriptional activation. It should be noted that our findings in this regard are in opposition to those of a recent study by Guzman et al. (12), in which initiation of rounds of chromosome replication were stimulated during a temperature shift of cells in which RNA synthesis activity was reduced. The investigators suggested that the displacement of RNA polymerase toward the heat shock genes reduced the transcription of a replication inhibition gene. We found no evidence of this phenomenon.

In our experiments, the cell cycle specificity of minichromosome replication was similarly affected whether overproduction of the DnaA protein was obtained by IPTG induction of the *ptac* promoter in plasmid pLSK5 at 30°C or upon thermoinduction of the p_L lambda promoter in plasmid pTAC1445. Furthermore, runout replication of chromosomes was similarly enhanced. On the other hand, minichromosome replication lasted for a longer period of time when DnaA overproduction was combined with a temperature upshift. Thus, the expression of DnaA-dependent initiation potential appears to be positively affected by an upshift in temperature, as observed previously by Xu and Bremer (41) for runout chromosome replication and Kornberg et al. (18) for *oriC* replication in vitro.

ACKNOWLEDGMENT

This work was partly supported by Public Health Service grant GM-26429 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. **Atlung, T., E. S. Clausen, and F. G. Hansen.** 1985. Autoregulation of the *dnaA* gene of *Escherichia coli* K12. Mol. Gen. Genet. 200:442–450.
2. **Atlung, T., A. Løbner-Olesen, and F. G. Hansen.** 1987. Overproduction of DnaA protein stimulates initiation of chromosome and minichromosome replication in *Escherichia coli*. Mol. Gen. Genet. 206:51–59.
3. **Atlung, T., K. V. Rasmussen, E. Clausen, and F. G. Hansen.** 1985. Role of the DnaA protein in control of DNA replication, p. 282–297. *In* M. Schaechter, F. C. Neidhardt, J. L. Ingraham, and N. O. Kjeldgaard (ed.), The molecular biology of bacterial growth. Jones and Bartlett Publishers, Inc., Boston.
4. **Baker, T. A., B. E. Funnell, and A. Kornberg.** 1987. Helicase action of dnaB protein during replication from the *Escherichia coli* chromosomal origin *in vitro*. J. Biol. Chem. 262:6877–6885.
5. **Baker, T. A., and A. Kornberg.** 1988. Transcriptional activation of initiation of replication from the *E. coli* chromosomal origin: an RNA-DNA hybrid near *oriC*. Cell 55:113–123.
6. **Baker, T. A., K. Sekimizu, B. E. Funnell, and A. Kornberg.** 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. Cell 45:53–64.
7. **Bramhill, D., and A. Kornberg.** 1988. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. Cell 52:743–755.
8. **Braun, R. E., K. O'Day, and A. Wright.** 1985. Autoregulation of the DNA replication gene *dnaA* in *E. coli* K-12. Cell 40:159–169.
9. **Frazier, M. W., and G. Mosig.** 1988. Roles of the *Escherichia coli* heat shock sigma factor 32 in early and late gene expression of bacteriophage T4. J. Bacteriol. 170:1384–1388.
10. **Fuller, R. S., B. E. Funnell, and A. Kornberg.** 1984. The dnaA protein complex with the *E. coli* chromosomal replication origin (*oriC*) and other DNA sites. Cell 38:889–900.
11. **Funnell, B. E., T. A. Baker, and A. Kornberg.** 1987. *In vitro* assembly of a prepriming complex at the origin of the *Escherichia coli* chromosome. J. Biol. Chem. 262:10327–10334.
12. **Guzman, E. C., A. Jimenez-Sanchez, E. Orr, and R. H. Pritchard.** 1988. Heat stress in the presence of low RNA polymerase activity increases chromosome copy number in *Escherichia coli*. Mol. Gen. Genet. 212:203–206.
13. **Hansen, F. G., S. Koefoed, L. Sørensen, and T. Atlung.** 1987. Titration of DnaA protein by *oriC* DnaA-boxes increases *dnaA* gene expression in *Escherichia coli*. EMBO J. 6:255–258.
14. **Helmstetter, C. E., and A. C. Leonard.** 1987. Coordinate initiation of chromosome and minichromosome replication in *Escherichia coli*. J. Bacteriol. 169:3489–3494.
15. **Helmstetter, C. E., and O. Pierucci.** 1976. DNA synthesis during the division cycle of three substrains of *Escherichia coli* B/r. J. Mol. Biol. 102:477–486.
16. **Koppes, L. J. H.** 1987. *oriC* plasmids do not affect timing of chromosome replication in *Escherichia coli* K12. Mol. Gen. Genet. 209:188–192.
17. **Koppes, L. J. H., and K. von Meyenburg.** 1987. Nonrandom minichromosome replication in *Escherichia coli* K-12. J. Bacteriol. 169:430–433.
18. **Kornberg, A., T. A. Baker, L. L. Bertsch, D. Bramhill, B. E. Funnell, R. S. Lasken, H. Maki, S. Maki, K. Sekimizu, and E. Wahle.** 1987. Enzymatic studies of replication of *oriC* plasmids, p. 137–149. *In* R. McMacken and T. J. Kelly (ed.), DNA replication and recombination. Alan R. Liss, Inc., New York.
19. **Kücherer, C., H. Lother, R. Kölling, M.-A. Schauzu, and W. Messer.** 1986. Regulation of transcription of the chromosomal *dnaA* gene of *Escherichia coli*. Mol. Gen. Genet. 205:115–121.
20. **Leonard, A. C., and C. E. Helmstetter.** 1986. Cell cycle-specific replication of *Escherichia coli* minichromosomes. Proc. Natl. Acad. Sci. USA 83:5101–5105.
21. **Lother, H., R. Kölling, C. Kücherer, and M. Schauzu.** 1985. *dnaA* protein-regulated transcription: effects on the *in vitro*

replication of *Escherichia coli* minichromosomes. EMBO J. 4:555–560.

22. **Matsui, M., A. Oka, M. Takanami, S. Yasuda, and Y. Hirota.** 1985. Sites of *dnaA* protein-binding in the replication origin of the *Escherichia coli* K-12 chromosome. J. Mol. Biol. **184:** 529–533.
23. **McMacken, R., L. Silver, and C. Georgopoulos.** 1987. DNA replication, p. 564–612. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
24. **Messer, W.** 1987. Initiation of DNA replication in *Escherichia coli*. J. Bacteriol. **169:**3395–3399.
25. **Messer, W., U. Bellekes, and H. Lother.** 1985. Effect of *dam* methylation on the activity of the *E. coli* replication origin, *oriC*. EMBO J. **4:**1327–1332.
26. **Neidhardt, F. C., and R. A. VanBogelen.** 1987. Heat shock response, p. 1334–1345. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
27. **Ogden, G. B., M. J. Pratt, and M. Schaechter.** 1988. The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell **54:**127–135.
28. **Pierucci, O., C. E. Helmstetter, M. Rickert, M. Weinberger, and A. C. Leonard.** 1987. Overexpression of the *dnaA* gene in *Escherichia coli* B/r: chromosome and minichromosome replication in the presence of rifampin. J. Bacteriol. **169:**1871–1877.
29. **Projan, S. J., S. Carleton, and R. P. Novick.** 1983. Determination of plasmid copy number by fluorescence densitometry. Plasmid **9:**182–190.
30. **Rokeach, L. A., A. Chiaramello, D. E. Junker, K. Crain, A. Nourani, M. Jannatipour, and J. W. Zyskind.** 1987. Effects of DnaA protein on replication and transcription events at the *Escherichia coli* origin of replication, *oriC*, p. 415–427. *In* R. McMacken and T. J. Kelly (ed.), DNA replication and recombination. Alan R. Liss, Inc., New York.
31. **Rokeach, L. A., and J. W. Zyskind.** 1986. RNA terminating within the *E. coli* origin of replication: stringent regulation and control by DnaA protein. Cell **46:**763–771.
32. **Russell, D. W., and N. D. Zinder.** 1987. Hemimethylation prevents DNA replication in *E. coli*. Cell **50:**1071–1079.
33. **Schauzu, M.-A., C. Kücherer, R. Kölling, W. Messer, and H. Lother.** 1987. Transcripts within the replication origin, *oriC*, of *Escherichia coli*. Nucleic Acids Res. **15:**2479–2497.
34. **Sekimizu, K., D. Bramhill, and A. Kornberg.** 1987. ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell **50:**259–265.
35. **Sekimizu, K., D. Bramhill, and A. Kornberg.** 1988. Sequential early stages in the *in vitro* initiation of replication at the origin of the *Escherichia coli* chromosome. J. Biol. Chem. **263:**7124–7130.
36. **Sekimizu, K., B. Y.-M. Yung, and A. Kornberg.** 1988. The dnaA protein of *Escherichia coli*: abundance, improved purification, and membrane binding. J. Biol. Chem. **263:**7136–7140.
37. **Smith, D. W., A. M. Garland, G. Herman, R. E. Enns, T. A. Baker, and J. W. Zyskind.** 1985. Importance of state of methylation of *oriC* GATC sites in initiation of DNA replication in *Escherichia coli*. EMBO J. **4:**1319–1326.
38. **Stark, M. J. R.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene **51:**255–267.
39. **von Meyenburg, K., and F. G. Hansen.** 1987. Regulation of chromosome replication, p. 1555–1577. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
40. **Wang, Q., and J. M. Kaguni.** 1987. Transcriptional repression of the *dnaA* gene of *Escherichia coli* by dnaA protein. Mol. Gen. Genet. **209:**518–525.

40a. **Weinberger, M., and C. E. Helmstetter.** 1989. Inhibition of protein synthesis transiently stimulates initiation of minichromosome replication in *Escherichia coli*. J. Bacteriol. **171:**3591–3596.

41. **Xu, Y.-C., and H. Bremer.** 1988. Chromosome replication in *Escherichia coli* induced by oversupply of DnaA. Mol. Gen. Genet. **211:**138–142.
42. **Zyskind, J. W., L. T. Deen, and D. W. Smith.** 1977. Temporal sequence of events during the initiation process in *Escherichia coli* deoxyribonucleic acid replication: roles of the *dnaA* and *dnaC* gene products and ribonucleic acid polymerase. J. Bacteriol. **129:**1466–1475.